

**POSTHARVEST IRRADIATION TREATMENT EFFECT ON GRAPEFRUIT
FUNCTIONAL COMPONENTS AND THEIR ROLE IN PREVENTION OF
COLON CANCER**

A Dissertation

by

JAIRAM KRISHNA PRASAD VANAMALA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2004

Major Subject: Horticulture

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ABSTRACT

Postharvest Irradiation Treatment Effect on Grapefruit Functional Components
and Their Role in Prevention of Colon Cancer. (August 2004)

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This dissertation examines the effects of postharvest treatment and processing on biologically active compounds of orange juice, and 'Rio Red' grapefruit and their ability to prevent chemically induced colon cancer in rat model. The first study evaluated the differences in flavonoid content of commercial 'made from concentrate' (MFC) orange juices and 'not from concentrate' (NFC) orange and grapefruit juices. Total flavonoid content of MFC orange juices (53 mg/100 mL; n = 12) was significantly ($P \leq 0.05$) higher than NFC orange juices (36.5 mg/100 mL; n = 14).

The second study investigated the ionizing radiation and storage effects on bioactive compounds and quality of "Rio Red" grapefruit. Results showed that storage and irradiation significantly ($P \leq 0.05$) affected the bioactive compounds in grapefruit, however, the effect of storage was prominent. The third study examined the influence of irradiation and freeze drying on bioactive compounds of grapefruit. Irradiation of grapefruit prior to freeze drying resulted in enhanced

($P \leq 0.05$) flavonoid content (naringin and narirutin). Freeze drying markedly reduced ($P \leq 0.05$) lycopene content. Freeze drying and irradiation reduced ($P \leq 0.05$) volatile compounds (d-limonene and myrcene), with the exception of ethanol.

In the fourth study suppression of colon cancer development in Sprague Dawley rats by natural and irradiated grapefruits and their functional compounds, naringin and limonin, were evaluated. The total number of aberrant crypts (AC; $P = 0.02$), number of high multiplicity AC foci (ACF; $P = 0.01$), and proliferative index ($P = 0.02$) were lower and apoptosis ($P = 0.02$) was higher in azoxymethane (AOM) injected rats on experimental diets. However, only natural grapefruit and limonin only suppressed AOM induced expansion ($P = 0.008$) of proliferative zone and also enhanced apoptosis more effectively than other experimental diets indicating that natural grapefruit and limonin may serve as better chemopreventive agents compared to IGFPP and naringin.

The present study indicates that postharvest quarantine doses of irradiation slightly alter composition of bioactive compounds and in turn marginally reduce the chemopreventive ability of grapefruit against the promotion stage of colon cancer. These results warrant the necessity of testing the impact of post harvest treatments on fruits and vegetables chemopreventive ability.

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CHAPTER I

INTRODUCTION

Multiple experimental and epidemiological studies over the past four decades have shown that diets rich in fruits and vegetables are protective against different cancers, including colon cancer (1). Colon cancer is the second leading cause of death from cancer in United States and the American Cancer Society estimates that there will be 106,370 new cases and 56,730 new deaths due to colon cancer in 2004 (2). Expansion of our knowledge on the role of dietary constituents in the prevention of colon cancer in recent years has provided the impetus for increasing emphasis on cancer prevention. Chemoprevention refers to the administration of chemical or natural agents that may block tumor initiation and/or promotion (3). It aims to decrease morbidity and mortality from colon cancer by lowering the risk of developing invasive or clinically significant disease. Chemoprevention is well suited for promotion stage of colon cancer because of its association with higher mortality, slow progression, and better understanding of its molecular pathogenesis.

The role phytochemicals in fruits and vegetables play in chemoprevention attracted researcher's attention since 1990. In recent years, phytochemicals are to find their proper place in health maintenance and disease prevention. Grapefruit are a very rich source of functional constituents, which may serve as chemopreventive agents (4), in addition to other beneficial effects on human

This dissertation follows the style and format of the Journal of Nutrition.

These constituents include flavonoids, limonoids, and their glucosides, vitamin C, folic acid, carotenoids (lycopene and beta-carotene), coumarin-related compounds (auraptene), high quality soluble fiber and potassium. In fact, a grapefruit phytochemical, limonin, has been found to significantly reduce the incidence of colonic adenocarcinoma induced by azoxymethane in male F344 rats (5).

The phytochemical content of fruits and vegetables is influenced by postharvest treatments and storage (6,7). Texas red grapefruits exported to international markets such as Japan and domestic markets such as Florida, California, and Arizona must be certified free of the Mexican fruit fly's maggots or larvae, *Anastrepha ludens* (Loew). Currently, methyl bromide fumigation is the most commonly used commercial quarantine treatment in Texas to overcome these trade barriers; however, it is toxic to humans and causes damage to the stratospheric ozone layer. Hallman et al. proposed low dose irradiation as an alternative to methyl bromide fumigation of 'Rio Red' grapefruit. However, low dose irradiation not only sterilizes the Mexican fruit fly larvae (8) but also influences the phytochemical content of citrus fruits (6,7,9), yet the impact of this treatment on the chemopreventive abilities of citrus fruits has not been examined in relation to colon cancer.

1.1 Background

1.1.1 *Colon cancer pathogenesis*

Colon cancer is a multi-step process reflecting genetic alterations that drive the progressive transformation of normal cells into highly malignant cells. Genetic alterations within the cell are initiated by the activation of a mutation in oncogenes or inactivation of tumor suppressor genes (10). These alterations often result in dysregulation of cell proliferation and/or apoptosis (11-13), which can lead to the formation of aberrant crypt foci (ACF).

Aberrant crypt formation is one of the best characterized markers for colorectal cancer in carcinogen induced animal models and involves expansion of the proliferative zone and inhibition of apoptosis (14). Azoxymethane (AOM), a commonly used colon carcinogen in animal models, can induce aberrant crypts during early stages of colon cancer as a consequence of extensive methylation of DNA bases, leading to a number of molecular mutations in regulatory genes consonant with the development of human colon cancer (15). Areas with four or more aberrant crypts are termed as high multiplicity aberrant crypt foci (HMACF). HMACF correlate particularly well with the incidence of colorectal adenomas and carcinomas in AOM-induced rat models (16).

Proliferation and apoptosis are the two most important players in the process of ACF formation. However, based on previous studies, it must be concluded that these markers for colorectal cancer are questionable diagnostic markers for tumor development, especially when assessed separately (17,18).

Yet, several studies conducted to test the chemopreventive efficacy of phytochemicals focused only on proliferation or apoptosis.

1.1.2 Grapefruit functional components

Evidence from a large number of epidemiological studies and animal studies has shown the consumption of citrus to be protective against a wide range of cancers in humans (1). Natural grapefruit juice suppresses carcinogen (PhIP) induced colon DNA damage (4). In 1992, the American Cancer Society (ACS) recognized citrus in the middle of the pyramid of cancer preventive fruits and vegetables. Initially it was assumed that vitamin C was the active agent in citrus as it is a free radical scavenger. However, research by Lam et al. (19,20) concluded that citrus contains not one but several anti-cancer agents.

1.1.3 Bioactivity of grapefruit functional components

Citrus flavonoids and limonoids may open a 'new avenue' to cancer prevention as they have repeatedly been shown to possess anticancer activity in animals (21-27). Research with animals and cancer cells grown in culture suggests that these chemicals are safe at reasonably elevated concentrations (28).

1.1.4 Flavonoids

Grapefruit and orange are rich sources of flavonoids, especially flavanones such as naringin, narirutin, and poncerin. Pharmacological properties of these flavonoids are linked to the ability of these compounds to promote differentiation (29), function as antioxidants (30), and alter the activities of a

number of intracellular enzymes including tyrosine kinases (31). The diverse effects of flavonoids may be due to their structural similarity to ATP, and hence, their ability to compete with ATP for binding at various enzymatic sites (32).

Grapefruits is rich in naringin, a flavanone glucoside, but naringenin, the aglycone of naringin is not abundant. However, Erlund et al. (33) reported relatively high concentrations of naringenin ($6.0 \pm 5.4 \mu\text{mol/L}$) in human plasma after the ingestion of grapefruit juice (8 mL/kg). These results suggest that naringin may be converted to naringenin in the intestine and then absorbed into the body. Like most flavonoids, naringenin has antioxidant and free radical scavenging properties (34,35) and has been reported to offer some protection against lipid peroxidation (35) and excessive cell proliferation (36). Recent studies show that naringenin suppressed the phosphoinositide 3-kinase (PI3K) activity in *adipose* cells (37). A large body of research data suggests that members of the PI3K family can also be considered as oncogenes because they control cell cycle progression, differentiation, survival, invasion, metastasis, and angiogenesis. Naringin also inhibits COX-2 activity, which plays a pivotal role in colon carcinogenesis (38). However, very little information is available on the effect of naringin on AOM-induced colon cancer in animal models.

1.1.5 Limonoids

Citrus limonoids are highly oxygenated triterpenoids and many are present (over 50 are known) in the Rutaceae and Meliaceae plant families. These furan ring-containing compounds are known to induce detoxifying

enzymes like glutathione-S-transferase (GST) in vital organs such as the liver (19). Limonin is the first compound characterized in this group of phytochemicals from citrus (39). It is abundant in young leaves and fruits when these tissues need protection from pathogen attack (31). These compounds were thought to be mere bitter components, until it was found that these compounds possess biological activity (40).

Limonin inhibited lung tumor formation in mice, and topical application of limonoids was found to inhibit both the initiation and promotion phases of carcinogenesis in the skin of mice (41). Furthermore, studies conducted in Japan showed that feeding orange juice containing flavonoids and limonoids inhibited AOM-induced colon cancer in male Fisher 344 rats (42). In a recent study, rats were injected with AOM (20 mg/kg body weight, once a week for 2 wk) and obacunone and limonin were fed in the diet at dose levels of 200 or 500 mg/kg for 4 wk during initiation or post-initiation. Both the citrus chemicals significantly inhibited ACF formation (55-65% reduction by 'initiation' feeding, 28-42% reduction by 'post-initiation' feeding). However, no differences were observed between rats fed with 200 or 500 mg/kg of limonin or obacunone (40).

1.1.6 Effect of low dose irradiation on grapefruit bioactive components

It appears that no detrimental effects on ascorbic acid, sugar, acid levels, or essential peel oil composition, after treatment of grapefruit with a dose of 300 Gy gamma irradiation (43). Sensory evaluation of juice from grapefruit treated with 750 Gy gamma irradiation showed that radiation treatment had no effect on

sensory qualities compared to storage (9). However, irradiation significantly increases the flavanone content of the Clementine orange (6,7). Currently very little information is available on irradiation effects on limonoids.

1.1.7 Effect of storage on grapefruit bioactive components.

Very little information is available for low temperature storage influence on grapefruit functional components. Generally, ascorbic acid content of fruits and vegetables tend to decline as the temperature or duration increases (44). However, limonoid and flavonoids content seems to increase with longer storage in grapefruit (45). Harvest time and other postharvest treatments also influence the bioactive component levels during storage. Higher doses of irradiation (400 Gy and 700 Gy) and 35 d of storage had detrimental effects on quality of early season grapefruit, however, no significant effect was observed on the quality of the late season fruit (45).

1.1.8 Effect of processing on functional components of grapefruit

Freeze drying has also been used extensively to dehydrate phytochemical-rich plants and test their chemopreventive ability in the animal models (46,47). Freeze drying has been reported to have no detrimental effect on total antioxidant activity of vegetables (broccoli, spinach, peas and sprouts) as determined by the Trolox equivalent antioxidant capacity (TEAC) assay (48). Some other study (49) has shown that plant products lose 12-30% of β -carotene due to freeze drying. However, information on freeze drying effects on bioactive compounds of grapefruits is elusive.

Different citrus flavonoids seem to respond differently to pasteurization and concentration processes. For example, didymin is sensitive to concentration process and its content in orange juices made from concentrate is generally lower than pasteurized 'not from concentrate' juices (50). Very little information is available on the influence of concentration and pasteurization processes on grapefruit flavonoid content.

1.1.9 Justification of rat model of human colon cancer

Aberrant crypt foci have been considered to be preneoplastic lesions in the colon of both humans (10) and experimental animals (51,52). The carcinogenic processes involved in the development of aberrant crypt foci are comparable between rat and humans in both proliferation and histological characteristics (53). The AOM-induced aberrant crypt foci model has previously been used by other scientists to evaluate the chemopreventive activity of citrus or citrus functional components (5,54).

In the light of above facts, we hypothesize that as compared to a basal diet, diets containing grapefruit or isolated bioactive compounds will result less ACF by decreased proliferation and increased apoptosis in AOM induced colon cancer in rats. To test this hypothesis, we evaluated the:

- Flavonoid content of commercial orange and grapefruit juices.
- Effect of irradiation, storage and/or freeze drying on grapefruit functional components such as flavonoids, terpenoids, limonoids and carotenoids.

- Formation of aberrant crypt foci in AOM-injected rats by microscopic examination (40X).
- Colonic proliferative index using PCNA assay and apoptosis by TUNEL assay.

CHAPTER II

VARIATION IN THE CONTENT OF BIOACTIVE FLAVONOIDS IN DIFFERENT BRANDS OF ORANGE AND GRAPEFRUIT JUICES

2.1 Synopsis

Citrus flavonoids have been shown to possess biological activities such as anti-inflammatory properties, cholesterol lowering and immune system modulation. In this study, 12 made from concentrate (MFC) and 14 not-from-concentrate (NFC) orange juices, and five NFC grapefruit juices available in the US market were analyzed for their flavonoid content by reverse phase HPLC. Individual and total flavonoid content was determined for all of the brands. The correlation between flavonoid content (mg) and price (US dollar) per unit volume of orange and grapefruit juices were also evaluated. Significant differences ($P \leq 0.05$) among the brands and within the brand were observed for all of the prominent flavanone glucosides. Within the brand, juice types containing added antioxidant vitamins C and E were not superior in flavonoid content compared to orange juice types devoid of added antioxidant vitamins. Total flavonoid content of MFC orange juices (53 mg/100 mL; $n = 12$) was significantly ($P \leq 0.05$) higher than NFC orange juices (36.5 mg/100 mL; $n = 14$). Hesperidin was found to be the major flavonoid followed by narirutin and didymin in orange juice. Naringin, narirutin, and poncerin were the major flavonoids in all brands of grapefruit juices. The concentration of didymin was considerably higher in NFC orange juices compared to MFC orange juices. Interestingly, no correlation was

observed between price and the total flavonoid content of MFC orange juices and NFC grapefruit juices. However, a significant negative correlation ($r = -0.49$; $P = 0.001$) was observed for NFC orange juices. This study provides valuable information on flavonoid composition of orange and grapefruit juices commonly available in the US market.

2.2 Introduction

Evidence from a large number of epidemiological, *in vitro* and *in vivo* studies has shown that consumption of citrus is protective against a wide range of cancers (27,42) and cardiovascular (55,56) diseases. Until the last two decades, it was assumed that vitamin C was the only chemopreventive agent in citrus because of its free radical scavenger ability. Recent accumulative evidences suggest that citrus contains several possible anti-cancer agents such as flavonoids and limonoids (5,27).

Orange and grapefruit are a rich source of health promoting flavonoids, especially flavanones, which are shown to possess several physiological properties. Flavonoids contain various glycosides of three main aglycones: hesperitin (4'-methoxy-3',4,7-trihydroxyflavanone), naringenin (5,7,4'-trihydroxyflavanone) and eriodictyol (5,7,3',4'-tetrahydroxyflavanone). Prominent glucosides in citrus are hesperidin and naringin, narirutin, and poncerin (50). Physiological properties of these flavonoids are attributed to their ability to inhibit cell proliferation, promote differentiation (29), function as antioxidants (30) and are modulators of tyrosine kinases (57). Flavonoids are

structurally similar to ATP and it is proposed that their ability to compete with ATP for binding at various enzymatic sites may be responsible for their varied biological effects (58).

For a long time, absorption of flavonoids from dietary sources was considered to be negligible. However, citrus flavonoids have been reported in plasma and urine of humans (33,59). Furthermore, recent reports suggest that humans absorb appreciable amounts of flavonoids in the small intestine (60) and citrus flavonoids could reach relatively high concentrations in human plasma after ingestion of orange or grapefruit juice (33).

Citrus juice consistently ranks first among the most consumed fruit juices in the US. On average, citrus juice consumption is 2 1/2 times greater than the second most preferred apple juice. Citrus juice consumption ($33.6 \text{ kg} \cdot \text{person}^{-1} \cdot \text{yr}^{-1}$) was much higher than the fresh citrus fruit consumption ($5.6 \text{ kg} \cdot \text{person}^{-1} \cdot \text{yr}^{-1}$) in the year 2000-01 (61). Orange juice producers utilize citrus juice concentrate or fruits from US, Mexico and Brazil. Citrus flavonoid content is influenced by the growing location (62). However, very little information is available on the variation in the flavonoid content in different brands of commercial orange and grapefruit juices available in the US market. This study investigated the variation in the major flavonoid content in different brands of orange and grapefruit juices and the correlation between the flavonoid content (mg), and unit price (US dollar).

2.3 Materials and Methods

2.3.1 Samples

Twelve MFC orange juices, 14 pasteurized NFC orange juices, and five NFC grapefruit juice products were analyzed (Tables 1, 2 and 3, respectively). A total of three samples (three replicates) were collected for each brand at monthly intervals from different grocery stores. In most cases, citrus juice container size was 1.89 L. Samples were frozen at -80°C until analysis.

2.3.2 Reagents and flavonoid standards

Dimethyl formamide (DMF) and acetonitrile (ACN) were obtained from VWR scientific products (Houston, TX). Naringin (Naringenine-7-rhamnosidoglucoside, NAR), narirutin (Naringenin-7-rutinoside, NAT), hesperidin (Hesperetin-7-rutinoside, HES), didymin (isosakuranetin-7-rutinoside, DID), neohesperidin (hesperitin 7-neohesperidoside, NEH), poncerin (Isosakuranetin-7-neohesperidoside, PON), kaempferol (3,5,7,4'-Tetrahydroxyflavone, KAP), rutin trihydrate (RUT), apigenin (5,7,4'-Trihydroxyflavone, APG), and quercetin dihydrate (3,5,7,3',4'-Pentahydroxyflavone dehydrate, QUE) were obtained from Indofine Chemical Company, Inc. (Hillsborough, NJ).

TABLE 1
MFC orange juices and their product label content, and source

Brand No.	Product name	Product labeled contents	Source
1	Albertson's ¹	Water, concentrated orange juice	U.S.A. and Mexico
2	Dole ²	100% pure orange juice from concentrate (filtered water and concentrated orange juice)	Brazil and U.S.A
3	Donald Duck ³	Water, concentrated orange juice, tricalcium citrate,	U.S.A., Brazil and Mexico
4	Good Day ⁴	Water, orange juice concentrate	U.S.A
5	Greater Value ⁵	Water, concentrated orange juice	U.S.A and Brazil
6	Greater Value with Calcium ⁵	Concentrated orange juice, calcium phosphate, calcium lactate	U.S.A and Brazil
7	Minute Maid Country Style ⁶	Pure filtered water, premium concentrated orange juice, orange pulp	U.S.A and Brazil
8	Minute Maid Home Squeezed plus Calcium ⁶	Pure filtered water, premium concentrated orange juice, orange pulp, Tricalcium phosphate, calcium lactate (calcium source)	U.S.A and Brazil
9	Minute Maid Premium Original ⁶	Pure filtered water, premium concentrated orange juice	U.S.A and Brazil
10	Minute Maid Premium Original plus Calcium ⁶	Pure filtered water, premium concentrated orange juice, Tricalcium phosphate, calcium lactate (calcium source)	U.S.A and Brazil
11	Minute Maid Premium Extra Vitamins Vit. C and E plus Zn ⁶	Pure filtered water, premium concentrated orange juice, ascorbic acid (vitamin C). Alpha-Tocopheryl Acetate (Vitamin E), Zinc gluconate (Zn source).	U.S.A and Brazil
12	Schepps ⁷	Water, orange juice concentrate	U.S.A

¹Albertson's Inc., General Office Boise, ID 83726.

²Dole, Duo Juice, Co. Bradenton, FL 34206.

³Donald Duck, Citrus World, Inc., Lake Wales, Florida 33853.

⁴Good Day, Albertson's Inc., Boise, ID 83726.

⁵Wal-Mart Stores Inc., Bentonville, AR 72716.

⁶The Minute Maid Company, A Division of the Coca-Cola.

⁷Schepps Dairy, Dallas, TX 75223.

TABLE 2
Commercial orange juices (NFC) and their product labels, and source

Brand No.	Product name	Product labeled contents	Source
1	Albertson's Country Style ¹	100% pasteurized orange juice. No water or preservatives added.	-
2	Albertson's Orange Juice plus Calcium ¹	100% pasteurized orange juice, Tricalcium citrate.	-
3	AvoClassic ²	Fresh squeezed orange juice.	Mexico
4	Florida Natural ³	Pasteurized orange juice.	U.S.A
5	H.E.B Calcium ⁴	Pasteurized orange juice, Tricalcium citrate.	U.S.A
6	H.E.B Groves Choice ⁴	Pasteurized orange juice.	U.S.A
7	H.E.B Home Squeezed Style ⁴	Pasteurized orange juice.	U.S.A
8	H.E.B Original ⁴	Pasteurized orange juice.	U.S.A
9	Horizon ⁵	Certified organic orange juice.	U.S.A and Mexico
10	Kroger Homestyle ⁶	Orange juice with orange pulp added.	Florida
11	Sam's Choice ⁷	Pasteurized orange juice.	U.S.A
12	Sam's choice with Calcium ⁷	Pasteurized orange juice, Calcium Phosphate, Calcium lactate, ascorbic acid (vitamin C).	U.S.A
13	Tropicana Pure Premium Calcium ⁸	100% pure squeezed pasteurized. orange juice, tangerine juice and FruitCal (calcium hydroxide, malic acid, and citric acid).	-
14	Tropicana Pure Premium Low Acid ⁸	100% pure squeezed pasteurized orange juice.	-

¹ Albertson's Inc., General Office Boise, Idaho 83726.

² AvoClassic, Keller, Texas 76248.

³ Florida's Natural Growers, Lake Wales, Florida 33853.

⁴ H.E.B, San Antonio, TX 78204.

⁵ Horizon Organic Dairy, Boulder, CO 80308.

⁶ Kroger Co., Cincinnati, Ohio, 45202.

⁷ Sam's Choice, Wal-Mart Stores Inc., Bentonville, AR 72716.

⁸ Tropicana Products, Inc., Bradenton, Florida 34206 USA.

TABLE 3

Commercial grapefruit juices (NFC) and their product labels, and source

Brand No.	Product name	Product labeled contents	Source
1	Albertson's Grapefruit Juice ¹	Ruby red grapefruit juice	-
2	Florida Natural Original ²	100% Pure	Florida
3	Horizon ³	100% pure squeezed pasteurized grapefruit juice	-
4	Kroger Grapefruit Juice ⁴	100% pure grapefruit juice, calcium citrate, (Vitamin C), calcium hydroxide, Vitamin D ₃	Florida
5	Tropicana Grapefruit Juice ⁵ plus Calcium	100% pure squeezed pasteurized ruby red grapefruit juice and FruitCal (calcium hydroxide, malic acid, and citric acid).	-

¹Albertson's Inc., General Office, Boise, ID 83726²Florida's Natural Growers, Lake Wales, Florida 33853³Horizon Organic Dairy, Boulder, CO 80308⁴Kroger Co., Cincinnati, Ohio, 45202⁵Tropicana Products, Inc., Bradenton, Florida 34206 USA

2.3.3 *Flavonoid analysis*

Commercial citrus juice samples were analyzed for flavonoid content by reverse phase liquid chromatography with modifications of the methods of Mouly et al. (50). Juice (20 mL) was homogenized with 20 mL of dimethylformamide and subsequently a 1.5 mL aliquot was centrifuged at 4000 g for 20 min. A 20 μ L sample was injected into the HPLC system (PE LC-250B and Model 200 Autosampler). Separation of flavonoid compounds was performed using a stainless-steel column (250 x 4.6 mm I.D.) packed with C₁₈ Altima, 5 μ m (Alltech, USA), equipped with a precolumn (7.5x4.6 mm I.D.) and a solvent system of acetonitrile (ACN)/water plus 4% acetic acid gradient starting at 0% and ending at 70% ACN concentration. The narirutin and naringin peaks were detected at 280 nm (PE-200 UV/VIS detector). Flavanones were identified by comparing their retention times with those of standards (Table 4). It is interesting that the retention times for narirutin, and naringin were not the same for both orange and grapefruit juice. This may be due to differential interaction of narirutin and naringin with other compounds in orange and grapefruit juices. Total flavonoid flavanone content was calculated by combining narirutin, hesperidin, didymin content and naringin, narirutin, poncerin, neohesperidin, quercetin content in orange and grapefruit juices, respectively.

2.3.4 *Statistical analysis*

Statistical analysis was performed using SAS (63). Significant differences between different brands of orange and grapefruit juices were tested by the

TABLE 4

Reference flavonoid standard's retention time (Rt)

Flavonoid	Rt (min)
Rutin	28.58
Neohesperidin	28.65
Eriocitrin	29.99
Narirutin	31.58
Naringin	33.35
Hesperidin	34.20
Neohesperidin	35.17
Quercetin	37.68
Didymin	41.97
Poncerin	42.31
Apigenin	47.30
Naringenin	47.63
Kaempferol	47.87

general linear model (GLM). Orange and grapefruit juice sample means were compared by the Tukey test at the 5% probability level. Pearson correlation was also calculated for total flavonoid content (mg) and unit price (US dollar).

2.4 Results and Discussion

The specific flavonoid groups analyzed were flavanones (naringin, narirutin, hesperidin, neohesperidin, didymin and poncerin) and flavonols (quercetin and rutin). Typical chromatograms obtained from orange juice and grapefruit juices are shown in Figures 1 and 2. It is clear from the chromatograms that hesperidin (peak 2; Fig. 1) and naringin (peak 2; Fig. 2) were the most predominant flavonoids in orange and grapefruit juice, respectively.

2.4.1 MFC orange juices

All individual flavonoids and the total flavonoid content were significantly different ($P \leq 0.05$) among some of the brands of MFC orange juices tested (Table 5). Hesperidin was the major flavonoid followed by narirutin and didymin and their concentrations ranged from 32.9 - 54.8, 4.4 - 8.0, and 1.17 - 2.57 mg/100 mL, respectively. The hesperidin levels reported are in agreement with previously reported values (50). Interestingly, variation in hesperidin content was greater compared to narirutin and didymin among the brands. Total flavonoid content ranged from 39.5 - 64.3 mg/100 mL, and was mainly influenced by the hesperidin content.

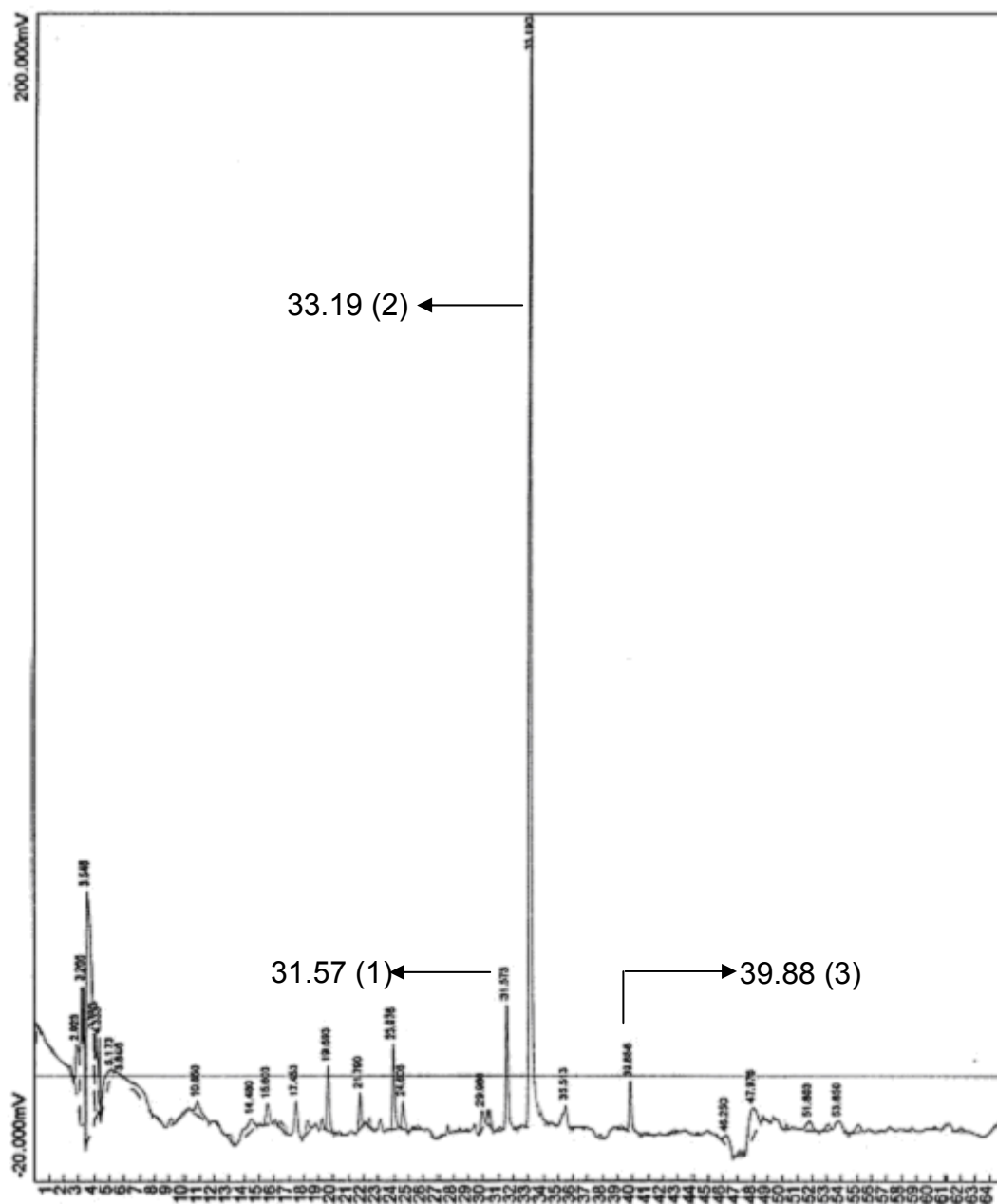


FIGURE 1 Chromatogram of orange juice sample. Peaks labeled 1, 2 and 3 (corresponding retention times clarified in call out boxes): 1, narirutin; 2, hesperidin and 3, didymin.

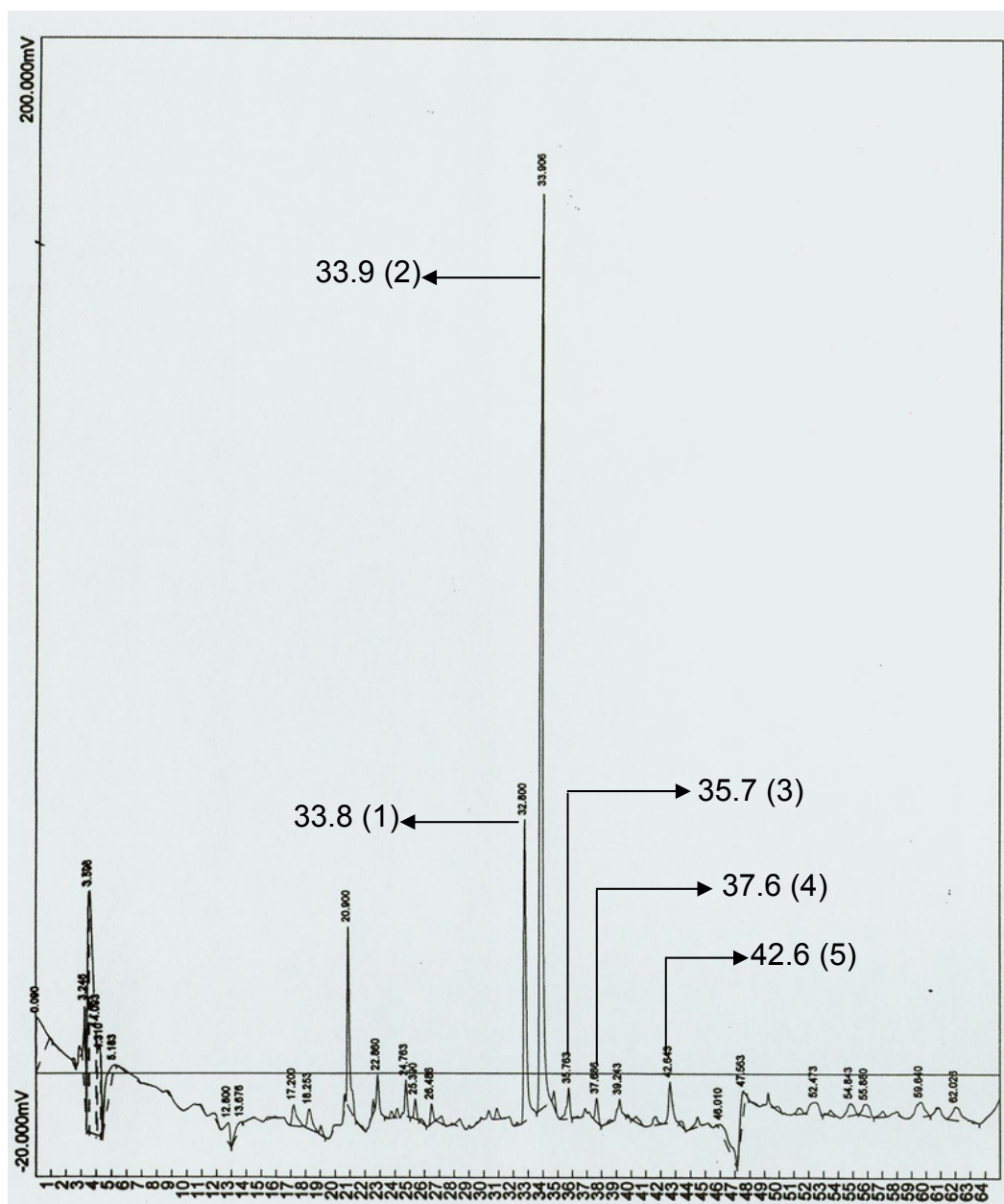


FIGURE 2 Chromatogram of grapefruit juice sample. Peaks labeled 1, 2, 3, 4 and 5 (corresponding retention times clarified in call out boxes): 1, narirutin; 2, naringin; 3, neohesperidin; 4, quercetin and 5, poncerin.

TABLE 5
Major flavonoid content of MFC orange juices

Brand name	Hesperidin (mg/100 mL)	Narirutin (mg/100 mL)	Didymin (mg/100 mL)	Total (mg/100 mL)
Albertson's	46.3 ^{abc1}	6.8 ^a	1.79 ^b	55.0 ^{abcd}
Dole	32.9 ^e	4.4 ^b	1.50 ^{bc}	39.5 ^e
Donald Duck	48.3 ^{abc}	7.1 ^a	1.80 ^b	57.2 ^{abc}
Good Day	34.0 ^{de}	8.0 ^a	1.74 ^b	44.3 ^{de}
Greater Value	44.3 ^{bcd}	6.6 ^a	1.83 ^b	52.8 ^{abcd}
Greater Value with Calcium	47.3 ^{abc}	6.8 ^a	1.17 ^c	55.3 ^{abcd}
Minute Maid Country Style	43.5 ^{bcd}	6.1 ^{ab}	1.50 ^{bc}	51.1 ^{bcde}
Minute Maid Home Squeezed plus Calcium	52.1 ^{ab}	7.8 ^a	1.91 ^b	61.7 ^{ab}
Minute Maid Premium Original	41.7 ^{bcde}	6.6 ^a	1.44 ^{bc}	49.7 ^{bcde}
Minute Maid Premium Original plus Calcium	43.2 ^{bcde}	7.0 ^a	1.63 ^{bc}	51.8 ^{bcde}
Minute Maid Premium Extra Vitamins Vit. C and E plus Zn	40.7 ^{edc}	6.4 ^{ab}	1.68 ^b	48.8 ^{cde}
Schepps	54.8 ^a	6.8 ^a	2.57 ^a	64.3 ^a
SEM	0.982	0.194	0.045	1.116

Data are means of three replicates for each brand.

¹Means within the column without the same letter are significantly different ($P \leq 0.05$; Tukey test).

Orange juices having added antioxidant vitamins such as vitamin C and E were compared to the juice types devoid of added antioxidant vitamins for the flavonoid content. Minute Maid Premium Extra containing vitamin C and E had significantly ($P \leq 0.05$) lower total flavonoid content compared to Home Squeezed plus Calcium. This may be due to the higher pulp content in the Home Squeezed plus Calcium brand compared to Minute Maid Premium Extra containing vitamins C and E. Overall Schepps had a higher content of hesperidin (54.9 mg/100 mL), didymin (2.57 mg/100 mL) and total flavonoids. However, Good Day had the highest narirutin content (8.0 mg/100 mL). Brands with lower hesperidin content did not necessarily have lower concentrations of narirutin and didymin. Within the Minute Maid brand different types of orange juices differed significantly ($P \leq 0.05$) in their flavonoid content and the differences were similar to the ones observed in Table 5 for that brand.

2.4.2 NFC orange juice

Pasteurized NFC orange juices also showed significant differences ($P \leq 0.05$) among individual flavonoid content and the total flavonoid content (Table 6). Hesperidin, narirutin, didymin and total flavonoid concentrations ranged from 18.0 - 42.8, 2.95 - 5.41, 1.16 – 3.14 and 23.5 - 50.4 mg/100 mL, respectively. These values are in agreement with another report (64). Hesperidin content variation appears to be greater in NFC orange juices than that of MFC orange juices. In general, hesperidin, narirutin, and total flavonoid content of NFC juices

TABLE 6
Major flavonoid content of 'NFC' orange juices

Brand name	Hesperidin (mg/100 mL)	Narirutin (mg/100 mL)	Didymin (mg/100 mL)	Total (mg/100 mL)
Albertson's Country Style	24.6 ^{fgh1}	3.64 ^{cde}	1.14 ^b	29.43 ^{ef}
Albertson's Orange Juice plus Calcium	39.1 ^{abc}	4.28 ^{bcd}	3.14 ^a	46.55 ^{abc}
AvoClassic	33.7 ^{dc}	3.57 ^{de}	1.58 ^{ab}	38.81 ^{cd}
Florida Natural	32.8 ^{cde}	4.58 ^{abc}	1.85 ^{ab}	39.21 ^{cd}
H.E.B Calcium	29.6 ^{defg}	4.33 ^{bcd}	1.76 ^{ab}	35.69 ^{de}
H.E.B Groves Choice	40.8 ^{ab}	4.68 ^b	2.36 ^{ab}	47.81 ^{ab}
H.E.B Home Squeezed Style	42.8 ^a	5.41 ^a	2.14 ^{ab}	50.36 ^a
H.E.B Original	30.7 ^{def}	4.08 ^{bcd}	2.12 ^{ab}	36.94 ^{de}
Horizon	18.0 ^h	4.34 ^{bcd}	1.24 ^b	23.53 ^f
Kroger Homestyle	23.8 ^{gh}	4.25 ^{bcd}	1.41 ^{ab}	29.41 ^{ef}
Sam's Choice	23.9 ^{fgh}	2.95 ^e	2.31 ^{ab}	29.12 ^{ef}
Sam's Choice with Calcium	34.8 ^{bcd}	3.78 ^{bcde}	1.16 ^b	39.78 ^{bcd}
Tropicana Pure Premium Calcium	26.2 ^{efg}	3.77 ^{bcde}	1.88 ^{ab}	31.86 ^{de}
Tropicana Pure Premium Low Acid	26.7 ^{efg}	3.97 ^{bcd}	1.70 ^{ab}	32.35 ^{de}
SEM	0.61	0.084	0.158	0.725

¹Means within the column without the same letter are significantly different (P ≤ 0.05; Tukey test).

were lower compared to MFC orange juices. Interestingly, didymin concentration was found to be higher in NFC orange juices compared to that of MFC orange juices. Mouly et al. (50) also reported numerically higher concentrations of didymin in NFC citrus juices than MFC orange juices. Gil-Izquierdo et al. (65) divided orange juice into a soluble fraction and cloud fraction (pulp), and observed that didymin was the most labile flavanone with a 52% loss in the soluble fraction of orange juice compared to the levels before concentration. However, other flavanones, such as hesperidin and naringenin showed only a slight decrease. Didymin was the most stable compound in the pasteurized orange pulp with a loss of only 1%. This may explain greater reduction of didymin content in the MFC orange juices compared to the pasteurized juices.

H.E.B Home Squeezed Style had a higher total flavonoid content among different brands of NFC orange juices. Significant differences in flavonoid content of both MFC and pasteurized NFC orange juices suggest that factors contributing to the variation need to be identified. These factors may be the growing conditions, storage, processing methods, and the differences in amount of water used during reconstitution of the orange concentrate to 'orange juice made from concentrate'.

2.4.3 Difference between MFC and NFC orange juices

MFC (n=12) and NFC (n=14) orange juices were compared for the total flavonoid content. Total flavonoid content was significantly ($P \leq 0.05$) higher in

MFC orange juices (53.2 mg/100 mL) compared to the NFC (36.49 mg/100 mL) orange juices (Fig. 3). Gil-Izquierdo et al. (65) reported that concentration doubled the total flavanone content in the cloud portion (pulp) due to the precipitation from the soluble fraction. Overall, it seems that concentration technique is not deleterious for the major bioactive flavonoids in the citrus fruit even though greater losses were observed in the didymin concentration.

2.4.4 NFC grapefruit juices

Naringin and narirutin content were significantly different ($P \leq 0.05$) among the brands tested (Table 7). However, no significant differences were found in the content of poncerin, neohesperidin and quercetin among the brands. Tropicana Grapefruit Juice plus Calcium recorded the highest total flavonoid content (52 mg/100 mL). Naringin and narirutin content in grapefruit juices ranged from 23.5 - 37.2 and 9.1 - 11.7 mg/100 mL, respectively. These results are in agreement with Patil et al. (66). However, Ross et al. (67) reported greater variation in naringin (14.6 – 51 mg/100 mL) and narirutin (2.25 - 11.40 mg/100 mL) contents compared to the values obtained in this study. The previous studies included juice types such as cocktails with only 35% fruit juice and also juices from concentrate. In contrast to previous work, the current study focused on 100% pure grapefruit juice. The low variation of flavonone content in our study may be due to use of only NFC grapefruit juice types.

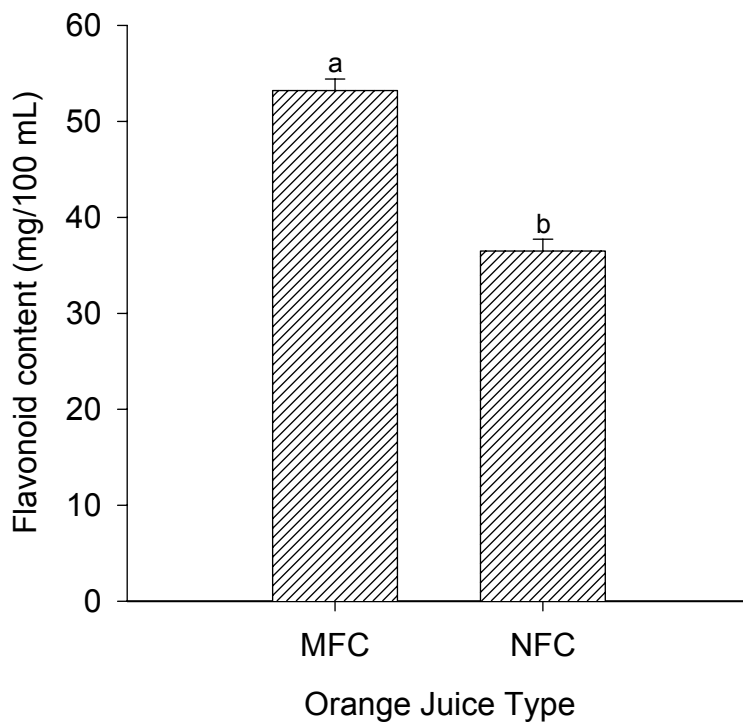


FIGURE 3 Total flavonoid content was significantly ($P \leq 0.05$) higher in made from 'concentrate' (MFC) orange juices ($n = 12$) compared to the 'pasteurized' NFC orange juices ($n = 14$).

TABLE 7
Major flavonoid content of 'NFC' commercial grapefruit juices

Brand name	Naringin (mg/100 mL)	Narirutin (mg/100 mL)	Poncerin (mg/100 mL)	Neohesperidin (mg/100 mL)	Quercetin (mg/100 mL)	Total (mg/100 mL)
Albertson's Grapefruit Juice	30.6 ^{b1}	10.4 ^{ab}	1.30 ^a	0.465 ^a	0.787 ^a	43.6 ^b
Florida Natural Original	31.5 ^b	10.2 ^{ab}	1.30 ^a	0.660 ^a	0.680 ^a	44.7 ^{ab}
Horizon	23.5 ^c	9.2 ^b	1.11 ^a	0.515 ^a	0.643 ^a	40.8 ^b
Kroger Grapefruit Juice	29.2 ^b	9.1 ^b	1.11 ^a	0.680 ^a	0.567 ^a	40.7 ^b
Tropicana Grapefruit Juice plus Calcium	37.2 ^a	11.7 ^a	1.37 ^a	0.620 ^a	0.505 ^a	52.2 ^a
SEM	0.769	0.262	0.088	0.054	0.070	1.239

¹Means with in the column without the same letter (a, b and c) are significantly different ($P \leq 0.05$; Tukey test).

2.4.5 Variation in total flavonoid content (mg) per US dollar among different brands of orange and grapefruit juices

Price did not correlate with the total flavonoid content ($r = 0.06$; $P = 0.759$) in MFC orange juice (Table 8). For example, Schepps had higher mean total flavonoid (64.307 mg/100 mL) content, however, Donald Duck had a higher flavonoid content per unit price (794.8 mg/US dollar; Fig. 4). NFC orange juices showed a negative correlation ($r = -0.49$; $P = 0.001$) between price and the total flavonoid content (Table 8; Fig. 5). Grapefruit juices ($r = -0.22$; $P = 0.426$) also showed similar trend as that of NFC orange juices however the correlation was not significant (Table 8; Fig. 6).

A study from England compared the costs and nutrient contents of five 'economy' line products of four major English supermarkets- Asda, KwikSave, Sainsbury, and Tesco with branded (but not 'own label') equivalents (68). This study also reported that the lower cost orange juices were nutritionally similar to and often better than the more expensive, branded citrus juices. People in the low-income group have the highest per capita consumption of orange juice . Consequently, information on the price and the levels of bioactive compounds would be beneficial for the consumer in choosing the best quality product for a given price, taking taste preferences into consideration. However, it is important to note that brand to brand differences identified in the flavonoid content in

TABLE 8

Pearson's correlation coefficient (r) between price and total flavanone content for commercial orange and grapefruit juices

Juice type	r-value	p-value
MFC Orange juice ¹	0.06	0.759
NFC orange juices ²	-0.49	0.001
NFC grapefruit juice	-0.22	0.426

¹MFC: Made from Concentrate

²NFC: Not from Concentrate

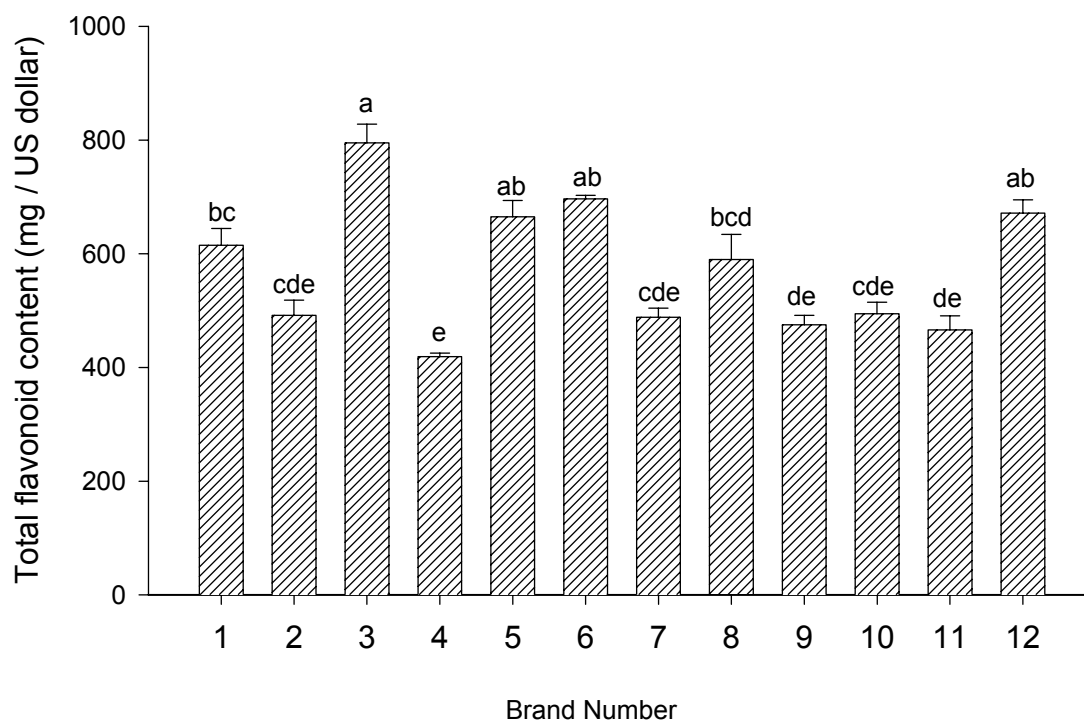


FIGURE 4 Variation in total flavonoid content (mg) per US dollar among different brands of MFC orange juice. Refer to Table 1 for a description of the orange juice brands.

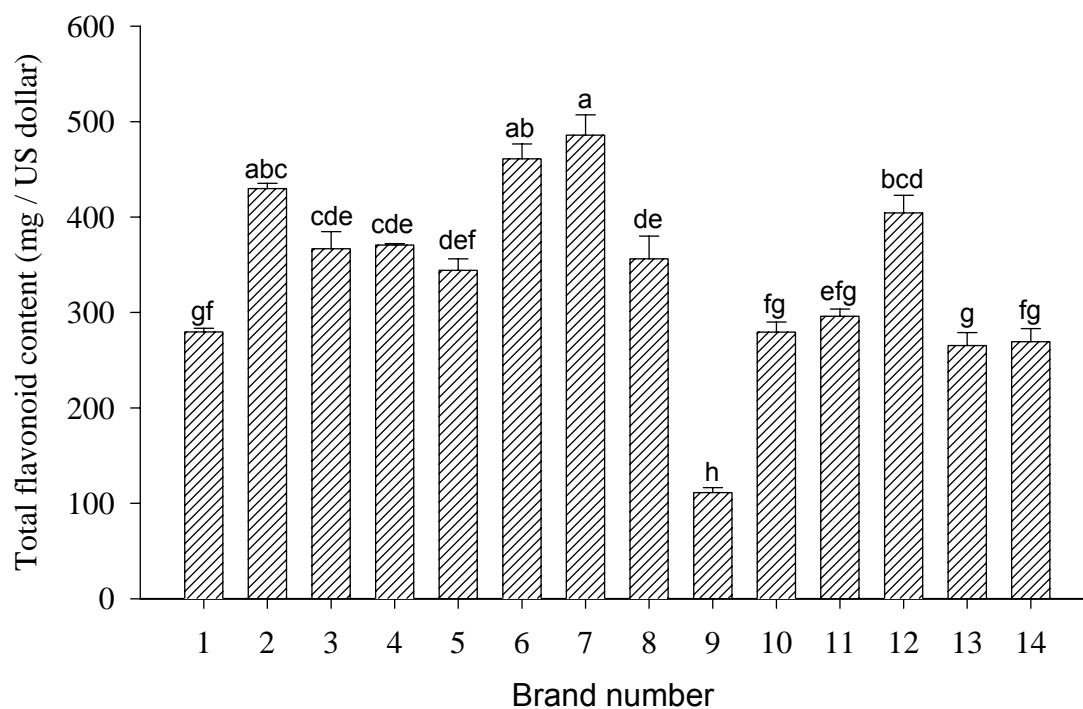


FIGURE 5 Variation in total flavonoid content (mg) per US dollar among different brands of NFC orange juice. Refer to Table 2 for a description of the orange juice brands.

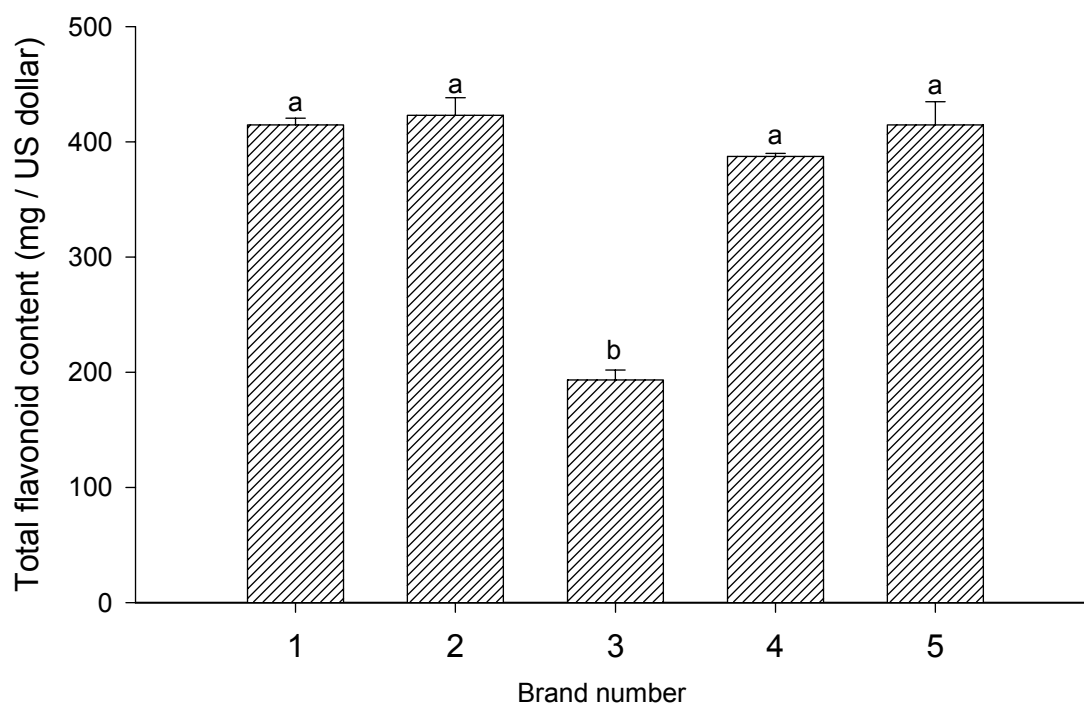


FIGURE 6 Variation in total flavonoid content (mg/ US dollar) of different brands of grapefruit juice. Refer to Table 3 for a description of the grapefruit juice brands.

one season may not be applicable to every season. Place of procurement or season may also influence the flavonoid content. For example, naringin content of grapefruit juice from the same grove and trees fluctuated during a season and varied considerably between crop years (69). The flavanone levels reported for juice brands tested in this study are applicable only to the time period the juices were tested.

2.4.6 Dietary intake of flavonoids

Dietary intake of flavonoid glucosides are reported to be 1000 mg/d when five classes (except bioflavonoid) of flavonoids were taken into consideration (70). According to the levels of hesperidin and narirutin obtained from MFC orange juices and pasteurized NFC orange juices, a 240 mL serving (Food and Drug Administration's standard serving size for fruit juices) would contain 43 - 132 mg of hesperidin and 7 - 19 mg of narirutin. A standard serving (240 mL) of orange juice provides 48 mg of flavanones (71). A similar volume of grapefruit juice could provide 58 - 89 mg of naringin and 22 - 29 mg of narirutin. It is evident that approximately 10-15% of Kuhnau's estimate of 1 g of daily intake of flavonoid glucosides could be obtained from one standard serving size of orange or grapefruit juice.

As humans are capable of rapidly absorbing flavonoid glucosides through the small intestine (72), a moderate or high consumption of orange juice or grapefruit juice, may result in citrus flavonoids being a significant part of the total polyphenolic pool in plasma (73).

Dietary intake of citrus flavonoids such as hesperidin and naringin may be protective against cardiovascular diseases because of their ability to reduce plasma and hepatic cholesterol, HMG-CoA reductase activity, Acyl CoA:cholesterol O-acyltransferase in rats, net apoB secretion in HepG2 cells and oxidation of LDL (74-76). These compounds were also shown to have protective effects against carcinogen-induced cancers such as skin, breast, esophagus, and colon in animal models (77-79). Even though the mechanism of action of flavonoids against carcinogenesis seems to be elusive, recent studies have worked on this area. Tanaka et al. (26) suggested that hesperidin decreased colon tumorigenesis in rats via an antiproliferative mechanism involving blockage of ornithine decarboxylase (ODC), in colonocytes exposed to a colon specific carcinogen.

Citrus flavonoids are potential antioxidants. Hesperidin effectively suppressed oxidative stress in diabetic rat (80). Hesperidin significantly reduced 8-hydroxydeoxyguanosine levels in the urine of streptozotocin-induced diabetic rats. Thus, citrus juice consumption on a regular basis may help protect individuals against major chronic disease such as cardiovascular diseases, cancer and diabetes.

It is evident from this study that commercially available orange and grapefruit juices differ in their bioactive flavonoids content. It would be interesting to evaluate the differences in other bioactive compounds in citrus fruits such as limonoids, carotenoids, ascorbic acid and potassium. Studies aimed to identify

the factors contributing to the changes in the bioactive compounds in citrus juices such as growing area; soil conditions, etc., are valuable in obtaining information which will help minimize the differences in bioactive compounds. It is possible that even though one bioactive compound is low in orange juice obtained from one type of growing conditions, other bioactive compounds may be higher. Thus, it is necessary to test different orange juices using experimental in vivo conditions to evaluate if the differences in bioactive compounds due to growing location and processing methods have a direct effect on their physiological properties.

CHAPTER III

IONIZING RADIATION AND STORAGE EFFECTS ON BIOACTIVE COMPOUNDS AND QUALITY OF “RIO RED’ GRAPEFRUIT

3.1 Synopsis

Irradiation is considered as an alternative method of quarantine treatment against fruit flies for citrus fruit as it also improves shelf life of the fruit during storage. Rio Red grapefruits (*Citrus paradisi* Macf.) were exposed to gamma irradiation from a ^{137}Cs source at 0, 150 and 300 Gy and then stored at 10°C for 36 d, followed by an additional 20 d at 20°C to simulate marketing conditions. Flavanone, terpenoid and quality (acidity and total soluble solids) were evaluated at regular intervals during storage. Results showed that irradiation and storage significantly ($P \leq 0.05$) affected the bioactive compounds in grapefruit, however, the effect of storage was prominent. Irradiation differentially effected the flavanone content of pulp and peel. For example, fruits exposed to 300 Gy had significantly ($P = 0.01$) higher narirutin content in peel compared to the fruits exposed to 0 Gy irradiation. However, narirutin content in pulp was not effected by irradiation. Even though storage enhanced the d-limonene and myrcene content in all treatments, control fruit had higher terpenoid content at the end of the storage. In general, irradiation or storage had no considerable effect on total soluble solids, however, acidity reduced ($P \leq 0.05$) with the storage and 300 Gy irradiated fruits better retained the acidity at the end of the storage.

3.2 Introduction

Fruit fly infestation is a world wide phenomenon with devastating effects on more than 100 fruit species, thus restricting food distribution. Common quarantine treatment for most fruits and vegetables against fruit flies is methyl bromide fumigation. However, methyl bromide is toxic to humans and has harmful effects on the ozone layer. In recent years, much effort is directed towards developing alternative methods to methyl bromide fumigation. Following the success in Hawaii of using low dose irradiation as a quarantine treatment against fruit flies (81), irradiation is being considered around the world as a potential alternative to methyl bromide (8,82). Ionizing radiation sterilizes or kills insects and microbial pests by damaging their DNA (83). In addition, low dose irradiation with γ -rays results in the intracellular generation of reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) in plant tissues, which may alter the phytochemical antioxidant content of fruits and vegetables (84).

'Rio Red' grapefruit are rich sources of bioactive compounds like ascorbic acid, flavonoids, and terpenoids that have been shown to reduce chronic diseases such as cancer (5). 'Rio Red' grapefruits from the Rio Grande Valley in Texas are exported mainly to California, Florida and Arizona. Exports account for 40% of market value and generate at least \$9.3 million/year for Texas grapefruit farmers (85). The farming community recognizes the need for developing an alternative quarantine treatment for fruit flies. A low dose of gamma irradiation as a quarantine treatment against fruit flies was recently

developed for citrus fruit (8). A minimum dose of 58 or 69 Gy was suggested for sterilization of fruit fly larvae; however, during commercial scale operations fruit could receive up to three times the minimum absorbed dose for quarantine purposes (8).

Even though several studies (8,43,86) have reported the effect of irradiation in the range of 150-300 Gy on citrus fruit quality parameters such as acidity, total soluble solids (TSS) and appearance, very little information is available on the effects of oxidative stress induced by irradiation and storage on grapefruit bioactive compounds like flavanones and terpenoids. This study investigates the effects of gamma irradiation and simulated storage on bioactive flavonoids, terpenoids and quality of grapefruit.

3.3 Materials and Methods

3.3.1 *Samples*

'Rio Red' grapefruit were collected from an orchard at the Texas A&M University-Kingsville, Citrus Center's South Farm, and the fruit were processed in a commercial packing line, washed and waxed.

3.3.2 *Irradiation treatment*

Irradiation treatments were carried out with a ^{137}Cs self contained dry-storage irradiator (Husman Model 521A, Isomedix, Inc., Whippany, NJ) at the USDA facility in Mission, TX. Fruit were irradiated at a dose of 150 Gy and 300 Gy with a centerline-absorbed dose of about 40 Gy min^{-1} . After the irradiation treatment, fruit were transported to College Station, TX and stored at 10°C for

36 d followed by an additional 20 d at 20°C to simulate routine marketing conditions. Pulp and peel samples were collected from six fruit at 12 d intervals (three samples) during low temperature storage followed by 10 d (two samples) during ambient temperature (20°C) and stored at -80°C until analyzed. Fruit samples were analyzed for flavanone content, terpenoids, total soluble solids (TSS), and titratable acidity.

3.3.4 *Flavanone analysis*

Grapefruit pulp samples were analyzed according to a modification of the method described by Mouly et al. (50) for flavanone content by reverse phase liquid chromatography. Fresh pulp (5 g) was homogenized with 20 mL (25 mL for peel) of dimethylformamide and subsequently a 1.5 mL aliquot was centrifuged at 7500 rpm for 20 min. This supernatant (20 µL) was injected into the HPLC system. Separation of flavonoid compounds were performed using a stainless-steel column (250 x 4.6 mm I.D.) packed with C₁₈ Altima, 5 µm (Alltech, USA), equipped with a precolumn (7.5x4.6 mm I.D.) and a solvent system of acetonitrile (ACN)/water plus 4% acetic acid gradient starting at 0% and ending at 70% ACN concentration. The flavanone peaks were detected at 280 nm. Flavanones were identified by matching their respective spectra and retention times with those of commercially obtained standards.

3.3.5 *Quantitative analysis of volatile grapefruit pulp terpenoid analysis*

Fresh grapefruit pulp (15 g) and 50 mL distilled water (24°C) were placed in a 540 mL plastic blender jar. The samples were blended with 200 µL of 5%

acetone, as an internal standard, for 2.5 min at medium speed using an Osterizer food blender. Headspace gas samples (1 mL) were injected into a GC (Perkin Elmer 8700 Model) equipped with a flame ionization detector (FID).

Operating conditions for the GC were: injector and detector temperature, 250°C; air and H₂ pressure, 138 and 105 kPa, respectively; and 30 mL/min of helium as carrier gas. A glass column (2 mm ID and 270 cm long) packed with 80% Carbowax 1500 on Chromosorb WAW-HMDS 80/100 mesh was used for the separation. Oven temperatures were maintained at 50°C for 0.5 min and raised to 130°C at the rate of 10°C/min (total run time of 8.5 min). Standard retention times were used to confirm the identity of volatile components.

3.3.6 Statistical analysis

The data was analyzed using a 3 x 6 factorial design with radiation dose and storage time intervals as factors and fruit weight as covariate in a GLM model of SAS (63). Treatment means were compared by LSD test at the 5% probability levels.

3.4 Results and Discussion

Fruits weight of grapefruit was significantly reduced with the storage time ($P = 0.001$) irrespective of the irradiation treatment (Fig. 7). Fruit exposed to 0 Gy irradiation dose had higher ($P = 0.008$) fruit weight compared to 150 Gy. No significant ($P \leq 0.05$) interaction between irradiation dose and storage time was

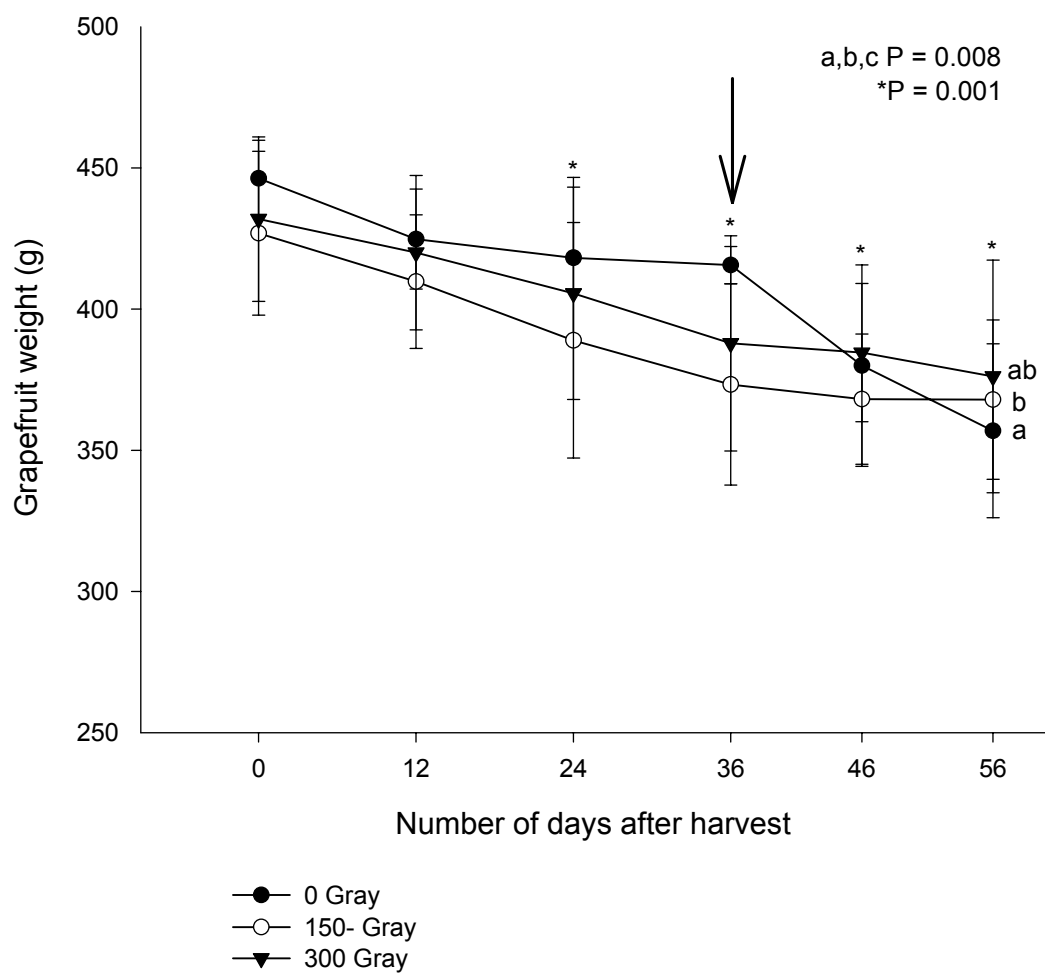


FIGURE 7 Irradiation and storage effects on fruit weight of ‘Rio Red’ grapefruit. *Indicates storage times which are significantly different from 0 d of storage regardless of irradiation dose. Same letter for the lines indicates no significant differences for irradiation dose regardless of storage. “Arrow” indicates time of transfer from low temperature (10°C) storage to ambient (20°C) conditions.

observed for fruit weight and any of the compounds tested in this study.

3.4.1 Influence of irradiation and storage on flavanone content

Flavonoids are polyphenolic compounds which are ubiquitous in plant cells. Flavonoids are categorized into flavonols, flavones, flavanones, isoflavones, catechins, and anthocyanidins. Flavanones are found abundantly in citrus fruit, mainly as flavonoid glycosides and are thus an important source of these compounds in the human diet (50). Flavonoids possess anticarcinogenic (53,87), antioxidant (88) and blood lipid lowering activities (75,89). However, little is known about irradiation and storage effects on bioactive flavonoids in the edible portion (pulp) and peel of 'Rio Red' grapefruit.

In our study, different flavanones in grapefruit peel and pulp responded differently to irradiation dose and storage. No interaction between irradiation and storage was observed for any of the compounds tested. Naringin content in grapefruit pulp was significantly ($P = 0.012$) lower in fruits exposed to 150 Gy compared to 0 and 300 Gy (Fig. 8). However, irradiation had no effect on naringin content of peel (Fig. 9). Naringin content in peel was significantly ($P = 0.028$) decreased at 12 d of storage compared to 0 d, however, no significant differences were observed for both peel and pulp at the end of the storage.

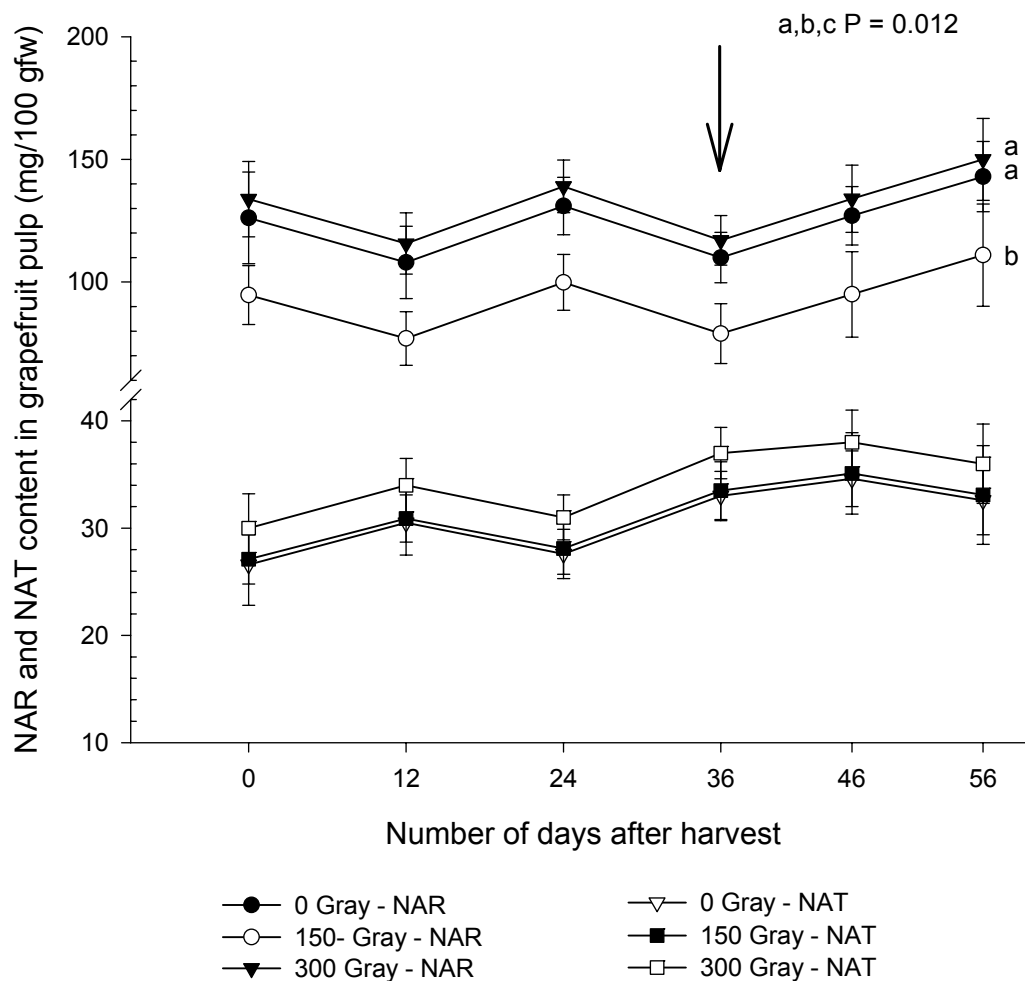


FIGURE 8 Irradiation and storage effects on naringin (NAR) and narirutin (NAT) content of 'Rio Red' grapefruit pulp. Same letter for the lines indicates no significant differences for irradiation dose regardless of storage. "Arrow" indicates time of transfer from low temperature (10°C) storage to ambient (20°C) conditions.

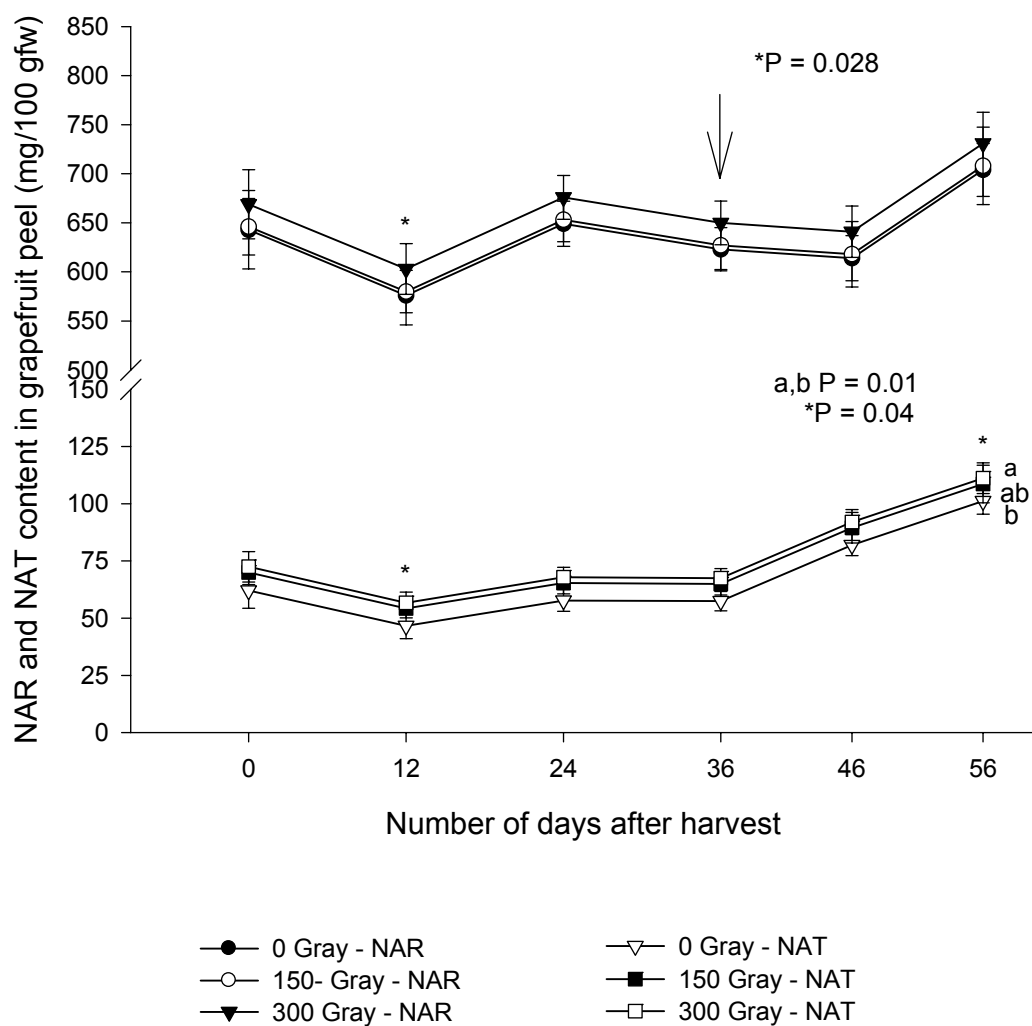


FIGURE 9 Irradiation and storage effects on naringin (NAR) and narirutin (NAT) content of 'Rio Red' grapefruit peel. *Indicates storage times which are significantly different from 0 d of storage regardless of irradiation dose. Same letter for the lines indicates no significant differences for irradiation dose regardless of storage. "Arrow" indicates time of transfer from low temperature (10°C) storage to ambient (20°C) conditions.

Narirutin content in pulp was not affected by irradiation or storage (Fig. 8). Fruits exposed to 300 Gy irradiation had significantly ($P = 0.01$) higher narirutin content in peel compared to the fruits exposed to 0 Gy of irradiation. However, peel narirutin content was significantly higher at the end of the storage compared to the 0 d eventhough significant ($P = 0.04$) decline was observed at 12 d of storage (Fig. 9).

Fruits treated with 150 Gy of irradiation had significantly lower poncerin content in pulp compared to the fruits exposed to 0 and 300 Gy (Fig. 10). However, fruits exposed to 0 Gy had significantly ($P = 0.026$) lower poncerin content in peel compared to fruits treated with 150 and 300 Gy (Fig 11). Poncerin content in pulp was not effected by the storage, however, a significant ($P = 0.016$) decline was observed in peel at 12 d of storage even though no significant differences were observed at the end of the storage compared to 0 d of storage.

Irradiation doses of 0 and 300 Gy resulted in significantly ($P = 0.02$) higher didymin content in pulp compared to 150 Gy (Fig. 10). However, didymin content in peel was significantly ($P = 0.001$) higher in fruits exposed to 300 Gy than the 0 Gy (Fig.11). Didymin content was significantly ($P = 0.03$) lower in pulp at 24 d and 46 d of storage compared to 0 d eventhough no significant differences were observed at the end of the storage. Interestingly, didymin content in peel increased ($P = 0.038$) with the storage.

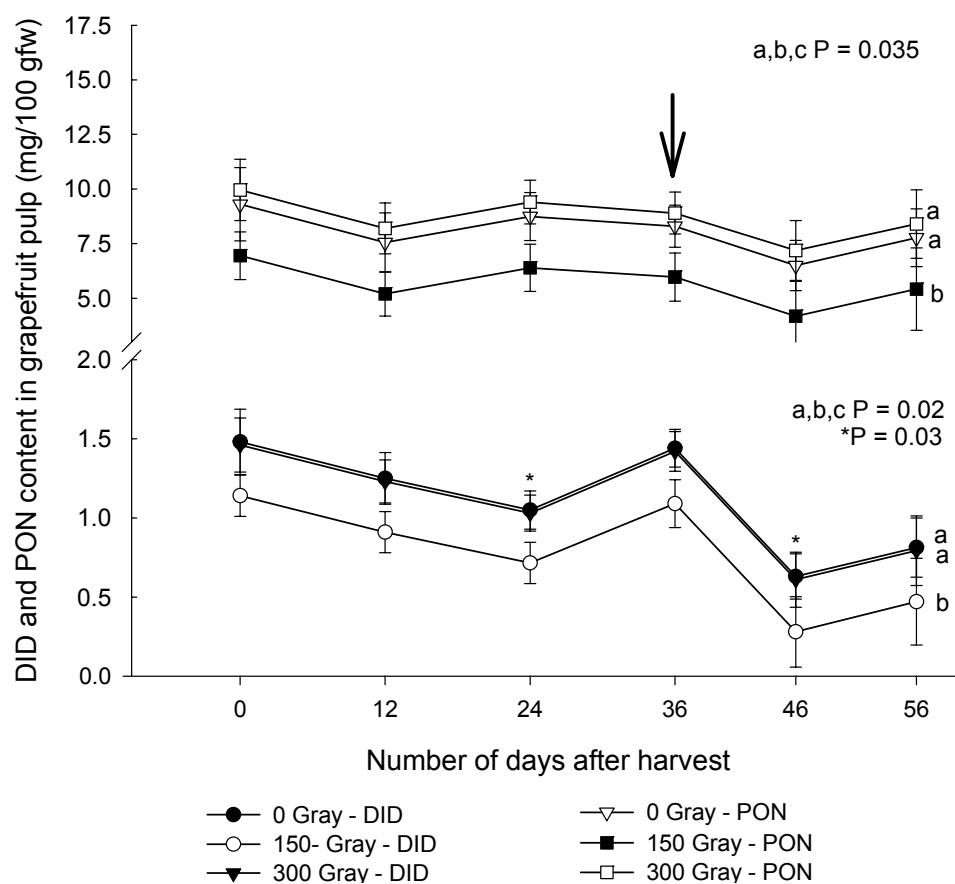


FIGURE 10 Irradiation and storage effects on didymin (DID) and poncerin (PON) content of 'Rio Red' grapefruit pulp. *Indicates storage times which are significantly different from 0 d of storage regardless of irradiation dose. Same letter for the lines indicates no significant differences for irradiation dose regardless of storage time. "Arrow" indicates time of transfer from low temperature (10°C) storage to ambient (20°C) conditions.

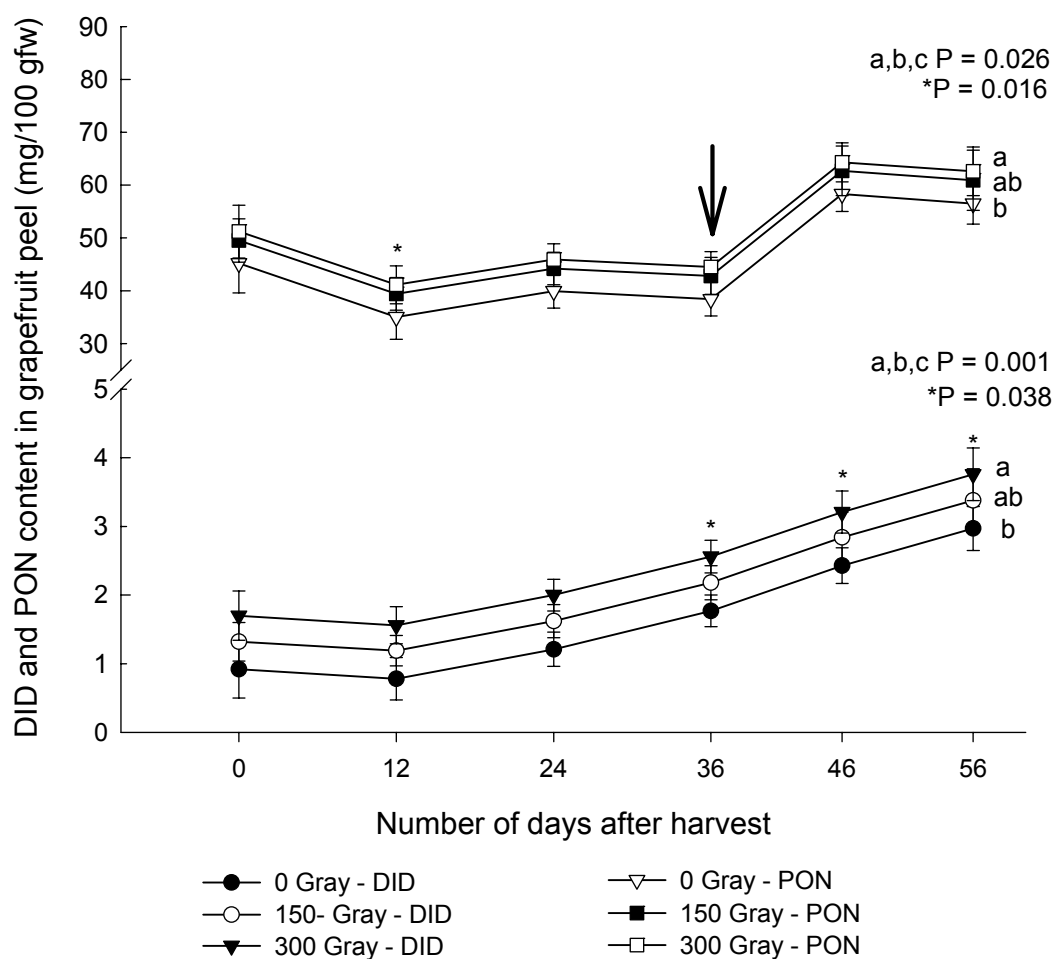


FIGURE 11 Irradiation and storage effects on didymin (DID) and poncerin (PON) content of 'Rio Red' grapefruit peel. *Indicates storage times which are significantly different from 0 d of storage regardless of irradiation dose. Same letter for the lines indicates no significant difference for irradiation dose regardless of storage time. "Arrow" indicates time of transfer from low temperature (10°C) storage to ambient (20°C) conditions.

Irradiation had no significant effect on both pulp and peel neohesperidin content (Fig. 12, 13). Storage also had no significant effect on neohesperidin content in the pulp. However, neohesperidin content was significantly ($P = 0.006$) increased in the peel at the end of the storage compared to 0 d.

Gamma radiation (90) and storage (7) has been found to stimulate phenolic biosynthesis in citrus fruit. PAL enzyme catalyzes the first reaction of the biosynthesis of flavanones and a large group of other phenolic compounds such as lignin's and coumarin in fruit (91). Previous research results suggests that temperature may be the regulator for stimulation or reduction of PAL activity (92). Furthermore, PAL activity was induced in Fortune mandarins stored at low temperature (93). Apples stored at 10°C had 2 times higher PAL activity as compared to that in apples stored at 24°C (94).

Some of the grapefruit pulp (naringin, poncerin and didymin) and peel (naringin, narirutin, poncerin and neohesperidin) flavanone content was decreased numerically or significantly ($P \leq 0.05$) after 12 d compared to 0 d at 10°C. Low temperature storage induces oxidative stress in fruit including citrus fruit (95). Rapid reduction in flavanone content at 12 d of storage may be due to rapid utilization of flavanones to scavenge reactive oxygen species produced by low temperature stress. Oufedjikh et al. (7) also reported a reduction in hesperidin (a flavanone) content in irradiated Clementine mandarin fruit at 7 d after low temperature storage.

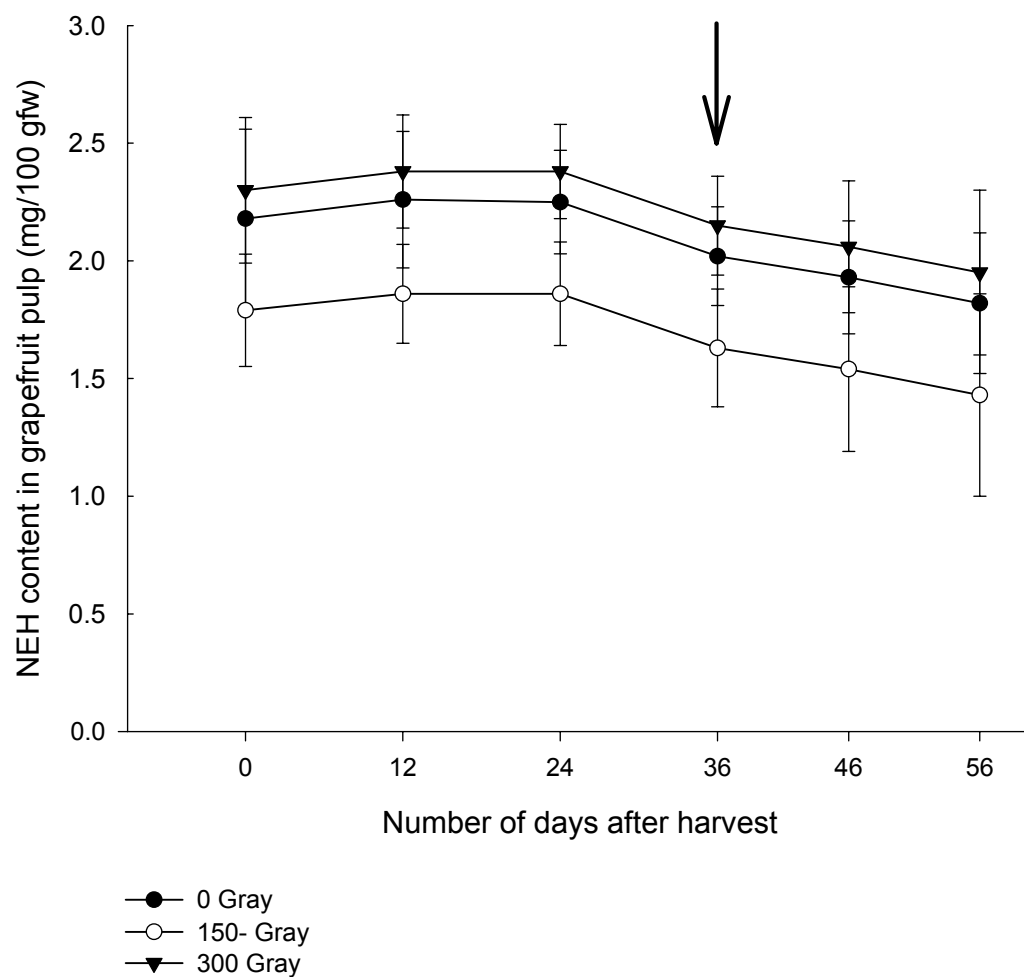


FIGURE 12 Irradiation and storage effects on neohesperidin (NEH) content of 'Rio Red' grapefruit pulp. "Arrow" indicates time of transfer from low temperature (10°C) storage to ambient (20°C) conditions.

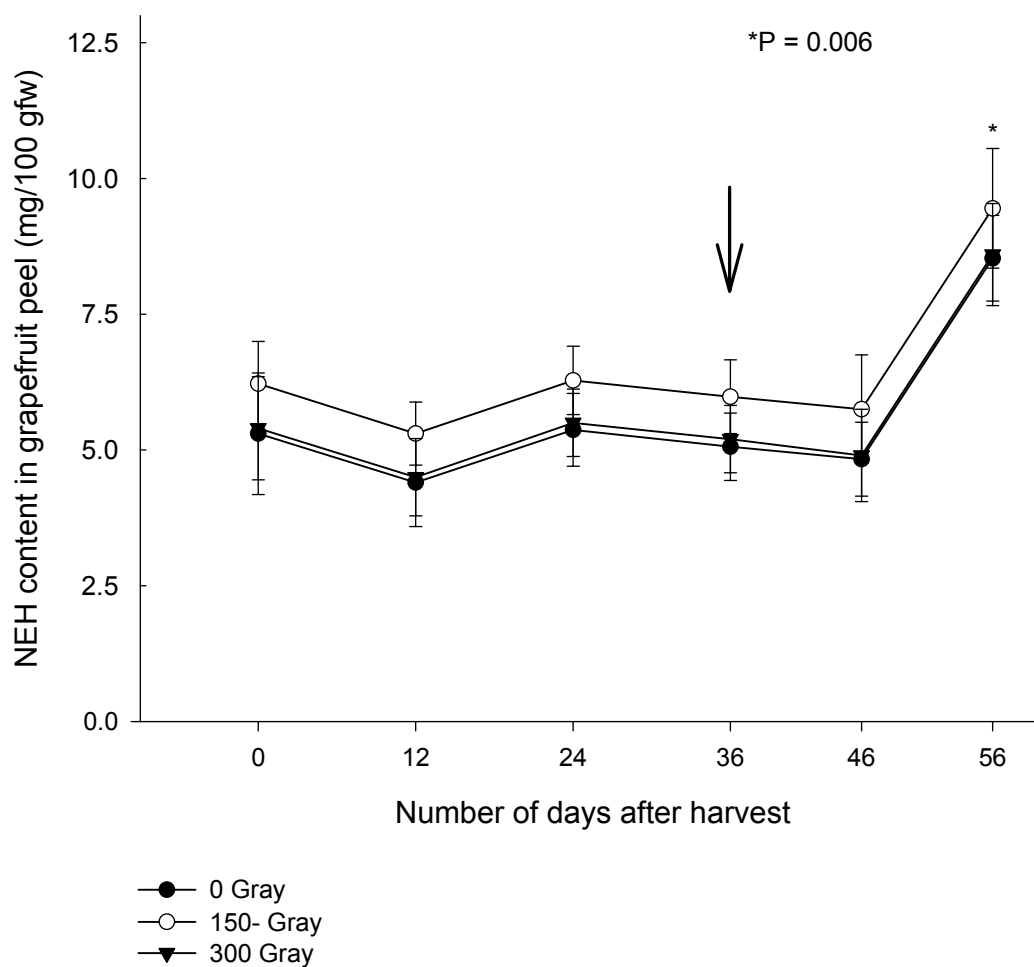


FIGURE 13 Irradiation and storage effects on neohesperidin (NEH) content of 'Rio Red' grapefruit peel. *Indicates storage times which are significantly different from 0 d of storage regardless of irradiation dose. "Arrow" indicates time of transfer from low temperature (10°C) storage to ambient (20°C) conditions.

Variation in flavanone content at different doses of irradiation treatment and time of storage may be due to equilibrium between gamma irradiation and low temperature induced oxidative stress and *de novo* synthesis of flavonoids by increased PAL activity (7).

3.4.2 Influence of irradiation and storage on terpenoid (d-limonene and myrcene) content

Terpenoids are the largest group of natural products in plants and several of them are biologically active (96). Monoterpenes such as d-limonene derived from citrus fruits have been shown to possess chemopreventive properties against mammary, liver, and/or lung carcinogenesis (97) .

d-Limonene and myrcene content in grapefruit pulp at the end of the storage was not significantly different from the 0 d of storage (Fig. 14 and 15). d-Limonene content was numerically lower at 12 d of storage compared to 0 d of storage. However, a gradual increase in d-limonene content was observed after 12 d of storage and the d-limonene content at the end of the storage was significantly ($P = 0.01$) higher compared to 12 d of storage. Non-irradiated (0 Gy) fruit had significantly higher d-limonene ($P = 0.005$) and myrcene ($P = 0.04$; Fig. 16) contents than fruits exposed to 150 Gy irradiation. Nunez-Selles et al. (9) reported numerical reduction in the d-limonene, myrcene and other volatile compounds in the irradiated (1000 Gy) grapefruit stored for 28 d at $12 \pm 1^{\circ}\text{C}$.

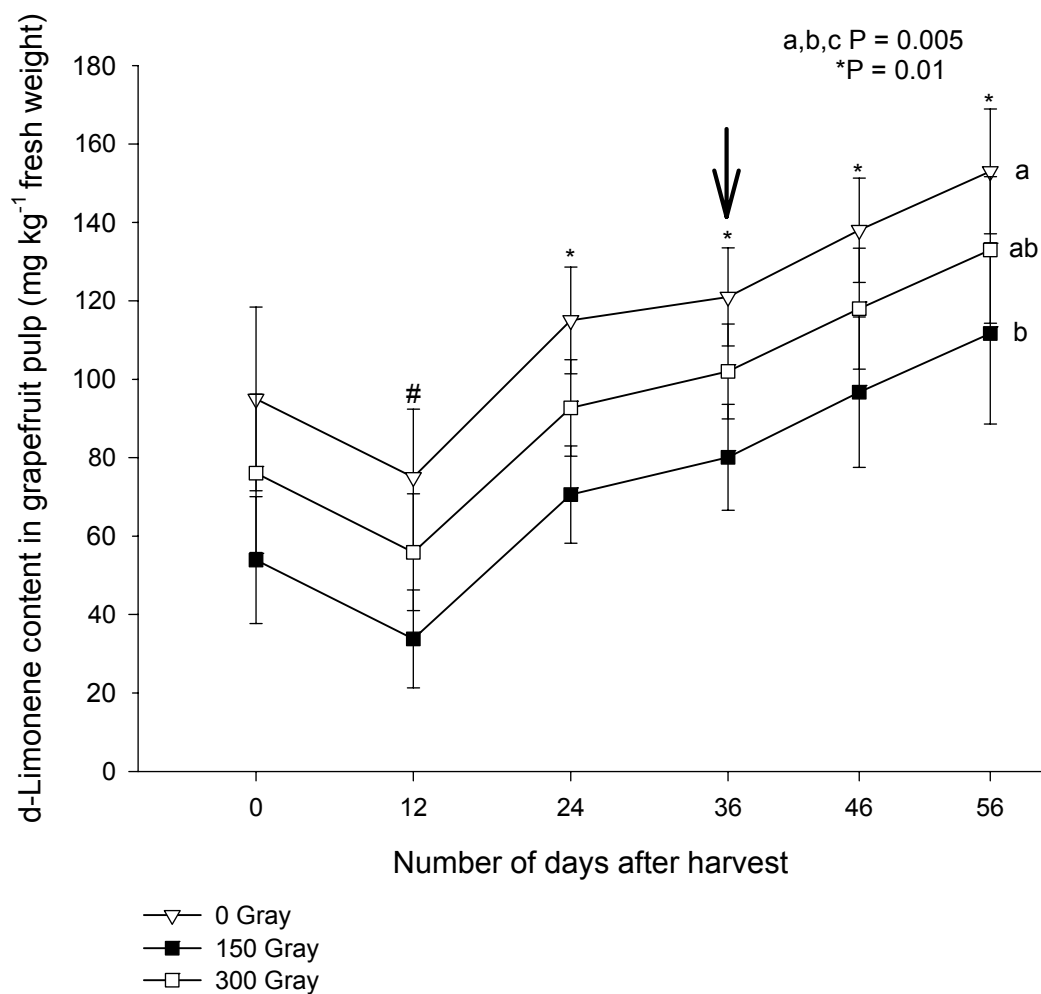


FIGURE 14 Irradiation and storage effects on d-Limonene content of ‘Rio Red’ grapefruit pulp. *Indicates storage times which are significantly different from 12 d (#) of storage regardless of irradiation dose. Same letter for the lines indicates no significant difference for irradiation dose regardless of storage time. “Arrow” indicates time of transfer from low temperature (10°C) storage to ambient (20°C) condition.

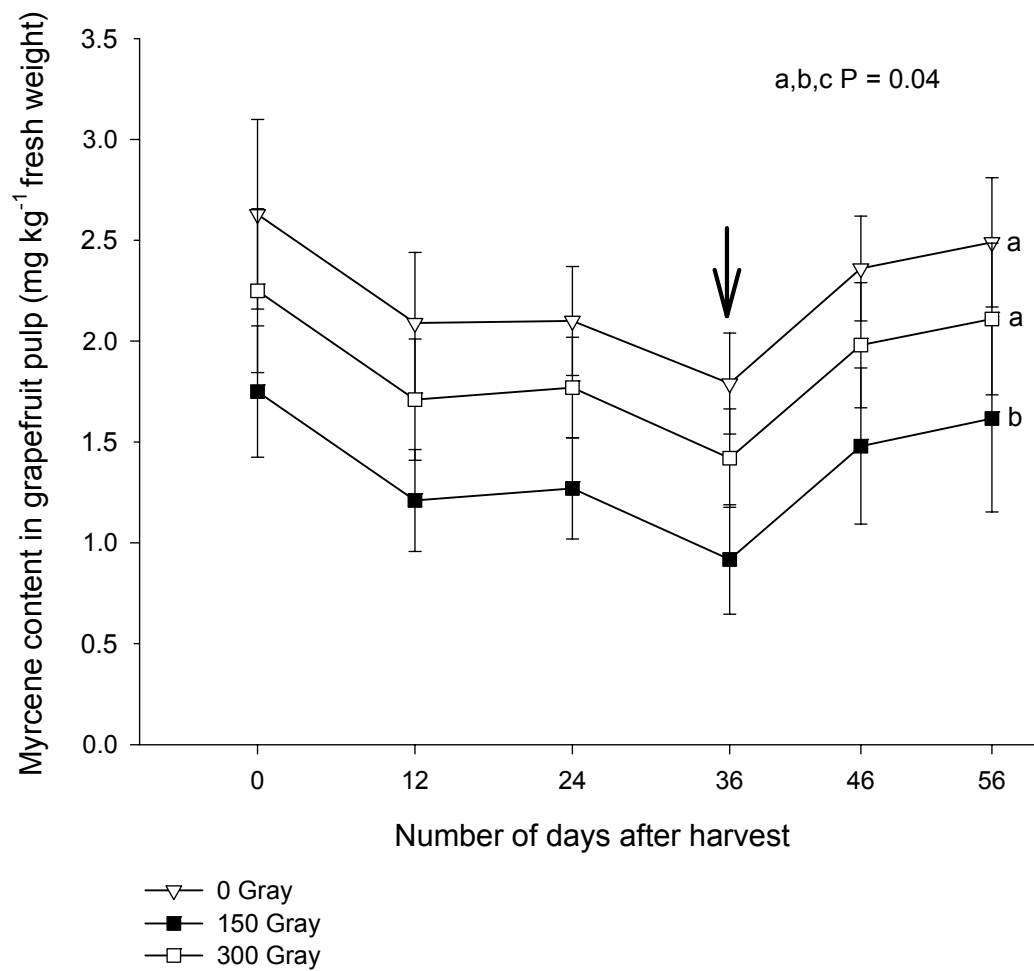


FIGURE 15 Irradiation and storage effects on Myrcene content of 'Rio Red' grapefruit pulp. Same letter for the lines indicates no significant difference for irradiation dose regardless of storage time. "Arrow" indicates time of transfer from low temperature (10°C) storage to ambient (20°C) conditions.

3.4.3 Irradiation and storage effect on grapefruit quality

Irradiation or storage did not result in considerable changes of the content of total soluble solids in grapefruits (Fig 16). However, there was a significant decline ($P = 0.009$) in acid content during storage (Fig. 16). Fruit respiration continues even at low temperature and organic acids are preferred substrates during respiration (98). Thus, reduction in acidity due to storage could be attributed to continued respiration during prolonged storage. Fruits exposed to 300 Gy of irradiation had significantly ($P = 0.012$) higher acidity compared to the control (0 Gy). Particularly at the end of the 60 d storage higher acid content was observed in asparagus irradiate at 1, 1.5 and 2 kGy (99).

In summary, our results suggest that low dose irradiation at 300 Gy enhanced or maintained the flavanone concentration in the pulp during storage and did not have deleterious effects on the quality. Our data suggests that irradiation at 300 Gy can be a viable quarantine treatment for grapefruit, as it promotes the content of functional constituents like flavanone and causes insignificant damage to the quality of grapefruit.

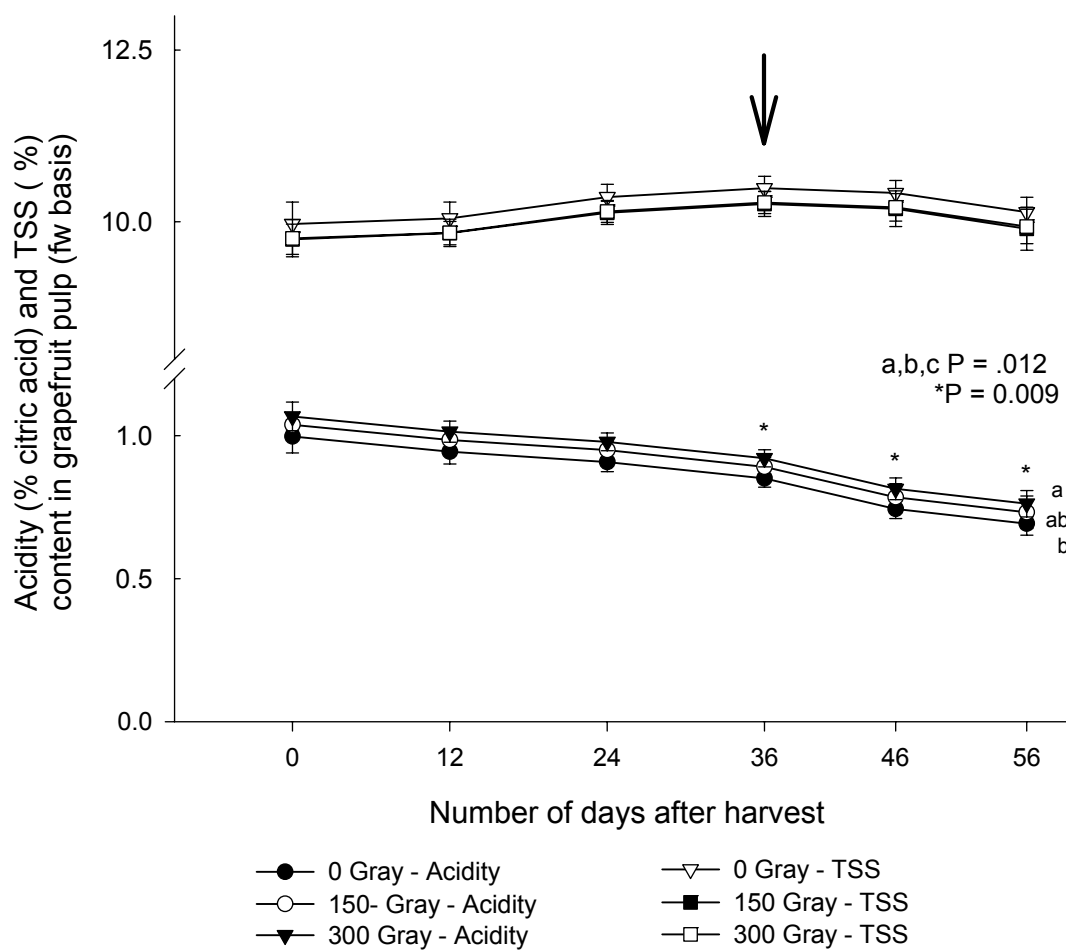


FIGURE 16 Irradiation and storage effects on acidity and TSS content of 'Rio Red' grapefruit pulp. *Indicates storage times which are significantly different from 0 d of storage regardless of irradiation dose. Same letter for the lines indicates no significant difference for irradiation dose regardless of storage time. "Arrow" indicates time of transfer from low temperature (10°C) storage to ambient (20°C) conditions.

CHAPTER IV

INFLUENCE OF IRRADIATION AND FREEZE DRYING ON BIOACTIVE COMPOUNDS OF GRAPEFRUIT

4.1 Synopsis

Bioactive compounds from fruits and vegetables are widely considered to be valuable for human health. However, the bioactive compounds of fruits and vegetables are influenced by postharvest treatments. This aspect has been mostly ignored or barely considered even in the contemporary nutritional and epidemiological studies due to limited scientific data on postharvest effects on bioactive compounds. The present study evaluated the effects of a proposed quarantine dose of irradiation (300 Gy), freeze drying and postharvest storage on bioactive grapefruit compounds such as flavonoids, carotenoids, ascorbic acid, limonoid aglycones, volatile compounds, and ascorbic acid. Bioactive compounds were analyzed with reverse phase liquid chromatography with the exception of volatile compounds which were analyzed using gas chromatography. Freeze dried pulp from irradiated grapefruit resulted in enhanced flavonoid content (naringin and narirutin). Freeze drying reduced ($P \leq 0.05$) grapefruit lycopene content, however, the reduction ($P \leq 0.05$) in β -carotene content was significant ($P \leq 0.05$) in control fruit only. Ascorbic acid was not affected by irradiation and the levels after storage were comparable to the day of harvest in control fruit. Freeze drying and irradiation (6 d) reduced volatile compounds (d-limonene and myrcene) with the exception of ethanol.

These results warrants the necessity of testing the effect of postharvest treatments like irradiation and processing effects on bioactive compounds in functional system as they have varied effects on different bioactive compounds of grapefruit.

4.2 Introduction

The role of phytochemicals in chemoprevention has been the subject of much research since 1990. In recent years, phytochemicals are gaining importance in health maintenance and disease prevention. Grapefruit are a rich source of bioactive phytochemical constituents such as flavonoids, limonoids and their glucosides, vitamin C, folic acid, carotenoids (lycopene and beta-carotene), coumarin-related compounds (auraptene), highly fermentable fiber and potassium. These constituents may serve as chemopreventive agents (100) in addition to other beneficial effects on human health. Citrus flavonoids (hesperidin and naringenin) and carotenoids (lycopene and beta-carotene) possess remarkable antioxidative activity. Limonin, another grapefruit phytochemical, has been found to significantly reduce the incidence of colonic adenocarcinomas induced by azoxymethane in male F344 rats (4).

Postharvest processes such as low dose irradiation (7) and freeze drying (101) can alter the phytochemical content of fruits. Currently irradiation is being considered as a versatile and viable alternative to toxic methyl bromide fumigation for treatment against Mexican fruit flies for Texas red grapefruit (8).

However, limited information is available on the effects of a quarantine dose of irradiation on the grapefruit bioactive compounds.

Freeze drying has also been used extensively to dehydrate phytochemical-rich plants and test their chemopreventive ability in the animal models (46,102). Several phytochemical rich freeze dried fruit and vegetable products are available as dietary supplements in the market, and the market for these kinds of products seems to be growing (103). However, the effect of postharvest processes such as low dose irradiation and freeze drying on bioactive phytochemical components is not well understood. This study aims to understand the impact of irradiation and freeze drying on grapefruit physiologically active bioactive components such as flavonoids, limonoids, volatile components, and carotenoids.

4.3 Materials and Methods

4.3.1 Samples

Three hundred and fifty 'Rio Red' grapefruit were collected from an orchard at the Texas A&M University-Kingsville Citrus Center's South Farm, and the fruit were processed in a commercial packing line, washed and waxed. One hundred and seventy five fruits were exposed to irradiation (300 Gy) and a similar number of fruits served as control. Samples were collected from six fruits on the day of harvest, and 4 and 6 d after irradiation. Grapefruit pulp, devoid of seeds, albedo and flavado was collected on the 7th day after irradiation from 150 fruits for freeze drying. Freeze drying was performed in six batches at NASA,

Shuttle and ISS Food Systems, Johnson Space Center, Houston, TX and samples were collected randomly from each replicate for analysis, and vacuum packed in Ziploc bags. All samples were stored at -80°C until analysis.

4.3.2 Irradiation treatment

Irradiation was carried out with ^{137}Cs self contained dry-storage irradiators (Husman Model 521A, Isomedix, Inc., Whippany, NJ) at the USDA facility in Mission, TX. Grapefruit were exposed to 300 Gy with a centerline-absorbed dose of about 40 Gy min^{-1} .

4.3.3 Flavanone analysis

Grapefruit pulp samples were analyzed for flavanone content by reverse phase liquid chromatography with modification of the procedure described by Mouly et al. (50). Fresh pulp (5 g) or freeze dried pulp (2 g) was homogenized with 20 mL (25 mL for peel) of dimethylformamide and subsequently a 1.5 mL aliquot was centrifuged at 7500 rpm for 20 min. This solution (20 μL) was injected into the HPLC system. Separation of flavanone compounds were performed using a stainless-steel column (250 x 4.6 mm I.D.) packed with C₁₈ Altima, 5 μm (Alltech, USA), equipped with a precolumn (7.5 x 4.6 mm I.D.) and a solvent system of acetonitrile (ACN)/water plus 4% acetic acid gradient starting at 0% and ending at 70% ACN concentration. The narirutin and naringin peaks were detected at 280 nm. Flavanones were identified by matching their respective spectra and retention times with those of commercially obtained standards from Indofine Chemical Company, Inc. (Hillsborough, NJ, USA).

4.3.4 Limonoid aglycone analysis

Freeze dried grapefruit pulp powder (3 g) of both control and irradiated fruits were homogenized with 50 mL of water and the pH was adjusted to 2.03-2.05. To this solution, 70 mL of ethyl acetate (0.7 M) was added and stirred thoroughly. Samples were centrifuged for 10 min at 9300 rpm. The supernatant was rotoevaporated to dryness and reconstituted with 2 mL methanol. The filtered samples (10 μ L) were injected into the HPLC. The analytical HPLC column for limonoid analysis was a C-18 reverse phase, Novapack column (Alltech Associates, Deerfield, IL), 4.6 x 250 mm, particle size 5 μ m and mobile phase was 10-50% acetonitrile plus 0.003M H₃PO₄. Limonoids were detected by UV absorption at 210 nm. Limonoids were identified by matching their respective spectra and retention times with those of external standards purified by the procedures perfected in our laboratory (104).

4.3.5 Terpenoid analysis

Fresh grapefruit pulp (15 g) or freeze dried pulp (2 g) samples and 50 mL distilled water (24°C) were placed in a 540 mL plastic blender jar. The samples were blended with 200 μ L of 5% acetone, as an internal standard, for 2.5 min at medium speed using an Osterizer food blender. Headspace gas samples (1 mL) were injected into a GC (Perkin Elmer 8700 Model) equipped with a Flame ionization detector (FID).

Operating conditions for the GC include injector and detector temperature, 250°C; air and H₂ pressure, 138 and 105 kPa, respectively; and 30

mL/min of helium as carrier gas. A glass column (2 mm ID and 270 cm long) packed with 80% Carbowax 1500 on Chromosorb WAW-HMDS 80/100 mesh was used for the separation. Oven temperatures were maintained at 50°C for 0.5 min and raised to 130°C at the rate of 10°C/min (total run time of 8.5 min). Standard retention times were used to confirm the identity of volatile components obtained from Sigma-Aldrich Corp., (St. Louis, MO, USA).

4.3.6 Carotenoid analysis

Samples of grapefruit pulp (5 g for fresh tissue and 1 g for freeze dried) were extracted with acetone (50 mL). This acetone extract was filtered using Miracloth™ filter and diluted with hexane (50 mL). At this point, double distilled water was added to this mixture (50 mL) and samples were stored in the dark for 24 h to allow the transfer of carotenoids into the hexane. Subsequently, aliquots (750 µL) of the hexane layer were transferred to an HPLC vial. Hexane was evaporated under helium gas and the carotenoids were resuspended in 750 µL of acetone. The HPLC apparatus of a PE LC-250B, equipped with series 200 autosampler and a UV-Vis detector was used. A 100 µL loop was used for injection. The mobile phases were acetonitrile:water (9:1; Solvent A) and ethyl acetate (35%; Solvent B). For both solvent A and B, 0.1% triethylamine was added to facilitate better interaction of mobile phase and stationary phase. The column was a C18 Spherisorb ODS-2, 5 m (250 × 4.6 mm i.d., Vydac) with a guard-column containing similar packing material (7.5 × 4.6 mm i.d., Alltech).

The column was kept at room temperature (about 22°C) with flow rate 1 mL·min⁻¹. The wavelength was adjusted to 450 nm.

4.3.7 Ascorbic acid analysis

Fresh (5 g) or freeze-dried pulp (2 g) was homogenized with 25 mL metaphosphoric acid (3%). A 1.5 mL aliquot was centrifuged at 7600 rpm for 20 min. The supernatant (20 µL) was injected onto a Waters Bondpak C-18 column (30 x 0.4 cm) with a guard column. The mobile phase was acetonitrile:water (70:30 v/v) with ammonium phosphoric acid (1.15 g/L) at the flow rate of 1.0 mL·min⁻¹. Ascorbic acid was detected at 255 nm with a run time of 60 min.

4.3.8 Statistical analysis

Statistical analysis was performed using SAS (63). This experiment utilized 2 x 4 factorial design. Significant differences between control and irradiated (2 factors) grapefruit bioactive compounds were evaluated over the storage time (4 factors) and their interaction using general linear model (GLM). Sample means were compared by the LSD test at the 5% probability level.

4.4 Results and Discussion

4.4.1 Influence of irradiation and freeze drying on flavanone content of grapefruit pulp

Naringin content increased in controls of fresh fruit after harvest (Fig. 17). When irradiated (300 Gy), naringin content was elevated over 0 Gray or at harvest 6 d after harvest and significantly ($P \leq 0.05$) higher compared to the irradiated fruits after harvest, 4 d after harvest and after freeze drying. Increased

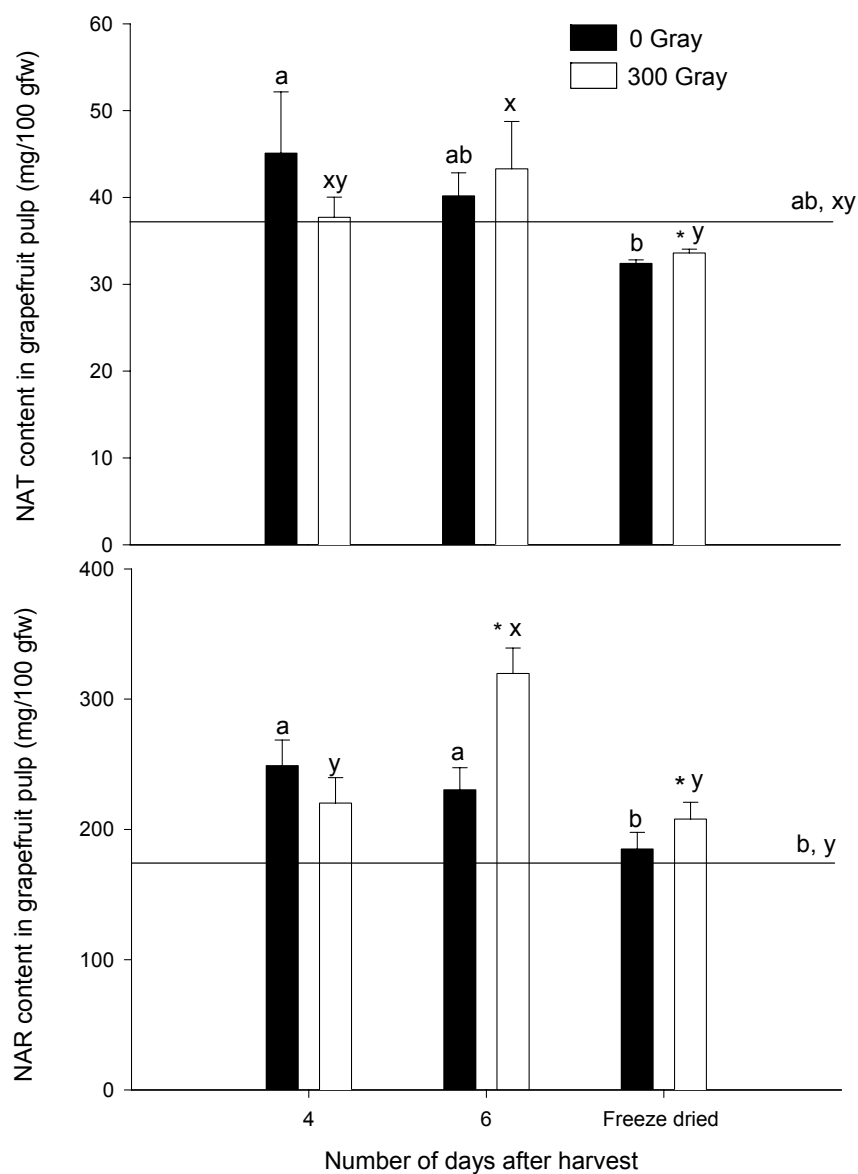


FIGURE 17 Irradiation and freeze drying effects on naringin (NAR) and narirutin (NAT) content of 'Rio Red' grapefruit (n = 6). *Indicates significant ($P \leq 0.05$) differences between the mean values of control and irradiated fruits at the same time interval. Same letter on the bar for control and irradiated fruits indicates no significant differences over time ($P \leq 0.05$). Horizontal line indicates the mean value on the day of harvest.

flavonoid content at 4 and 6 d of storage compared to the levels at harvest in control fruits may be because of enhanced phenylalanine ammonia-lyase (PAL) activity (105,106). PAL is a crucial enzyme for the biosynthesis of flavonoids. PAL catalyzes the deamination of L-phenylalanine to form trans-cinnamic acid, a precursor for flavonoids and tannins (91). Irradiation has also been shown to induce PAL activity in variety of fruits including citrus fruit (7,107). These results suggest that stress induced by harvest in combination with irradiation synergistically increases PAL activity.

This, stress induced *de novo* synthesis of naringin may counteract the degradation shown to occur with the freeze drying process (108). While naringin and narirutin contents decreased due to freeze drying, their contents were comparable to the day of harvest. Narirutin ($P \leq 0.05$) and poncerin ($P \leq 0.05$) declined significantly in the freeze dried control samples compared to the levels 4 d after harvest. Previous reports suggest that flavanone glucosides such as naringin and narirutin are sensitive to freeze drying (108). However, the naringin and narirutin content in freeze dried controls are not significantly ($P \leq 0.05$) different from the day of harvest, since its content was elevated after harvest due to stress induced PAL activity. Didymin and neohesperidin were not affected by irradiation, freeze drying or the number of days after harvest (Fig. 18 and 19). The differential response of these flavonoids to irradiation could be attributed to differences in the structure of the flavonoids.

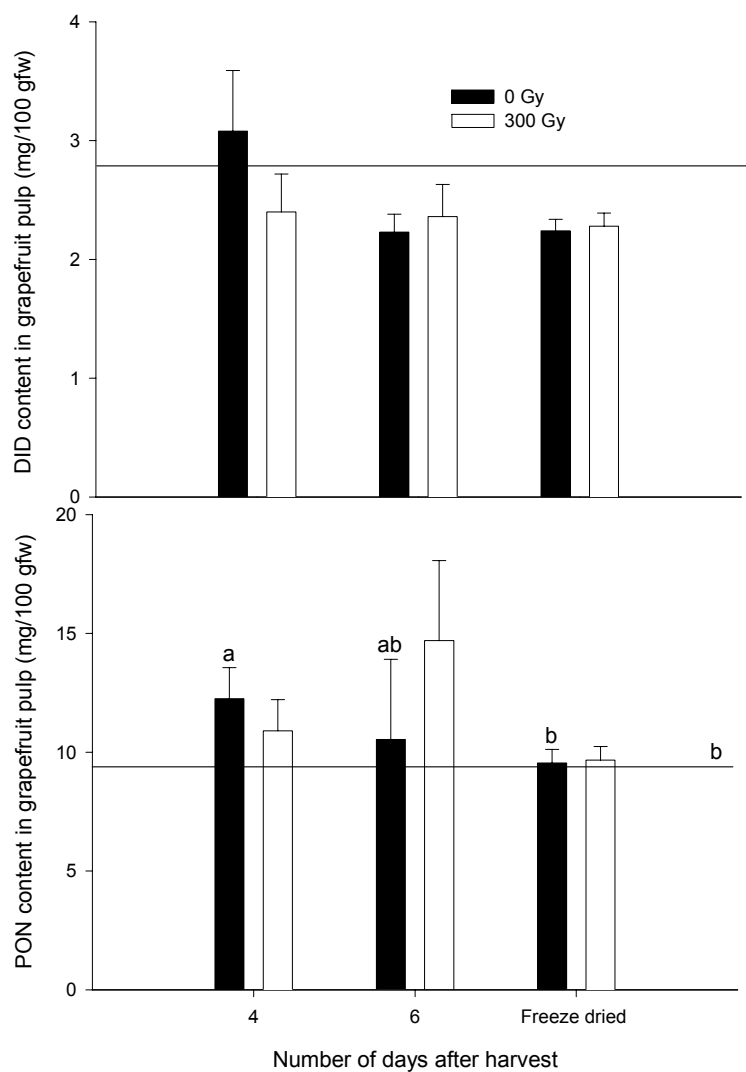


FIGURE 18 Irradiation and freeze drying effects on poncerin (PON) and didymin (DID) content of 'Rio Red' grapefruit (n = 6). Same letter on the bar for control fruits indicates no significant differences across time ($P \leq 0.05$). Horizontal line indicates the mean value on the day of harvest.

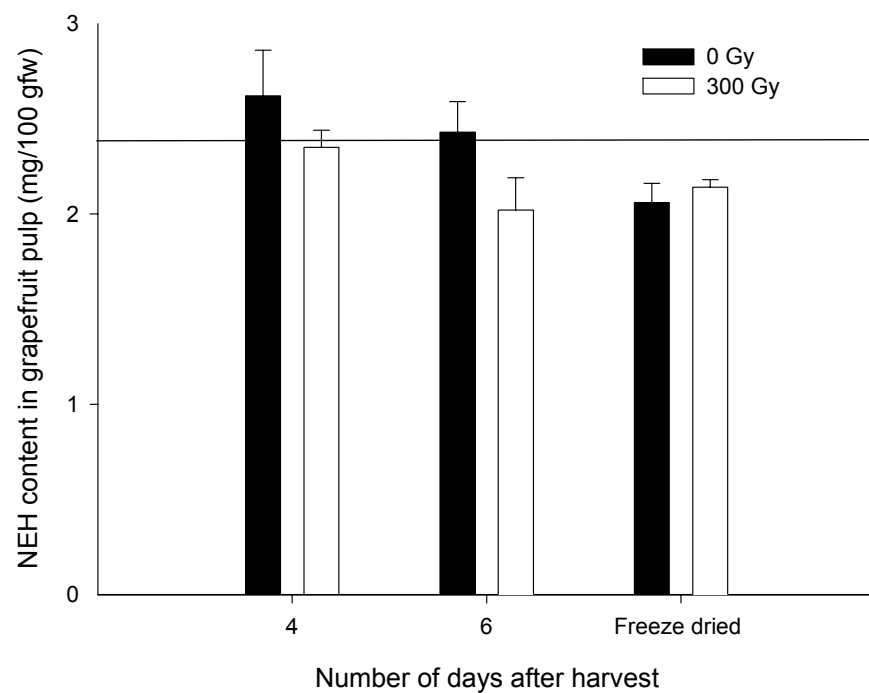


FIGURE 19 Irradiation and freeze drying effects on neohesperidin (NEH) content of 'Rio Red' grapefruit ($n = 6$). Horizontal line indicates the mean value on the day of harvest.

It is also evident from the other studies that some flavonoids are not altered by irradiation (108), freeze drying (108) or storage (45).

4.4.2 Influence of irradiation and freeze drying on lycopene, β -carotene and ascorbic acid content of grapefruit pulp

Lycopene content in freeze dried fruits was less ($P \leq 0.05$) than the fruits at the day of harvest (Fig. 20). However, freeze dried pulp from irradiated fruit had significantly higher lycopene content compared to control fruit. Similar results were also seen for β -carotene in control fruits. Freeze dried pulp from irradiated fruits also showed a reduction in β -carotene content compared to the day of harvest, but it was not significant. Grapefruit carotenoids, especially lycopene, appear to degrade when the fruit pulp or juice is extracted from the fruit (109). It is a possibility that lycopene may be converted to other isoforms. Previous studies (49,110) have also shown that plant products lose 12-30% of β -carotene due to freeze drying. However, our previous studies showed numerically higher β -carotene content in grapefruit exposed to gamma radiation (45). This may explain why irradiated freeze dried pulp did not show a significant difference in the β -carotene content compared to the fruits on the day of harvest, as at 4 and 6 d after harvest.

Interestingly, ascorbic acid content was not significantly affected by the irradiation treatment (Fig. 21). Several studies have shown that loss of vitamin C in citrus is minimal up to doses of 1000 Gy (43). Freeze drying significantly ($P \leq$

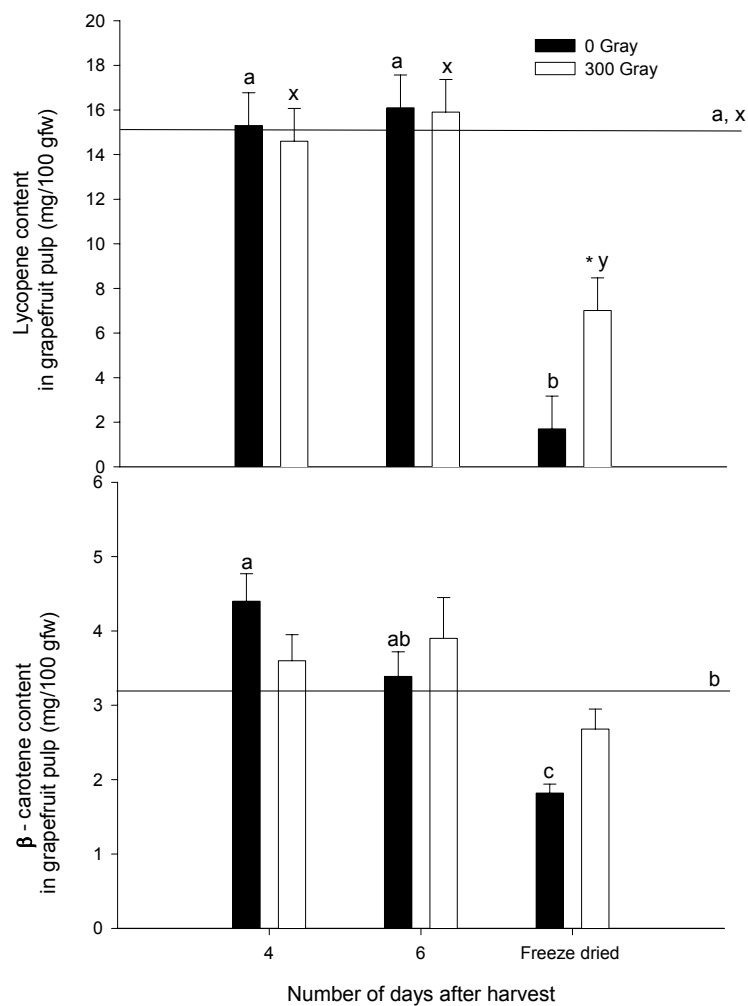


FIGURE 20 Irradiation and freeze drying effects on β - carotene and lycopene content of 'Rio Red' grapefruit (n = 6). Same letter on the bar for control and irradiated fruits indicates no significant differences over time ($P \leq 0.05$). Horizontal line indicates the mean value on the day of harvest.



FIGURE 21 Irradiation and freeze drying effects on ascorbic acid content of 'Rio Red' grapefruit (n = 6). Same letter on the bar for irradiated fruits indicates no significant differences over time ($P \leq 0.05$). Horizontal line indicates the mean value on the day of harvest.

0.05) reduced the ascorbic acid content in irradiated grapefruit pulp powder, however, the reduction was not significant in control fruit. Minimal loss of ascorbic acid was also recorded in natural strawberries after freeze drying (101).

4.4.3 Influence of irradiation and freeze drying on volatile compounds

The content of d-limonene in the freeze dried pulp of both control and irradiated fruits were significantly less ($P \leq 0.05$) than the day of harvest or at 4 d and 6 d after harvest (Fig. 22). While there was no difference in control fruits at 4 and 6 d after harvest, irradiated fruits at 6 d after harvest had less ($P \leq 0.05$) d-limonene content than irradiated fruits at 4 d and immediately after harvest. Furthermore, myrcene was reduced to non-detectable levels in the freeze dried fruits. Bos et al. (111) also reported a significant reduction in volatile compounds of cow parsley, *Anthriscus sylvestris* (L.) Hoffm after freeze drying. Freeze drying of parsley also resulted in a marked decrease in the majority of volatile compounds (112). Myrcene content was significantly lower in irradiated fruit at 4 and 6 d after harvest compared to immediately after harvest. Nunez Selles et al. (113) also reported a reduction in the volatile compounds including d-limonene and myrcene in irradiated fruits.

Freeze dried pulp from irradiated grapefruit had significantly higher ($P \leq 0.05$) ethanol content compared to freeze dried control and compared to the irradiated fruits at the day of harvest, or at 4 and 6 d after harvest (Fig. 22). However, ethanol content in irradiated fruit is significantly ($P \leq 0.05$) lower than

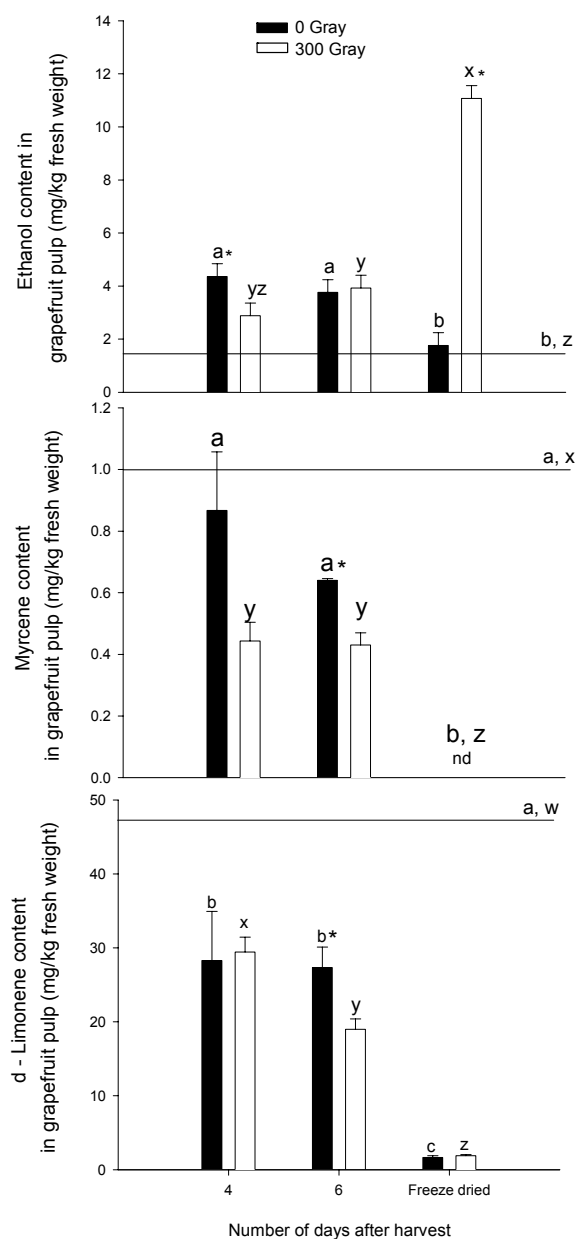


FIGURE 22 Irradiation and freeze drying effects on d-limonene, myrcene, and ethanol content of 'Rio Red' grapefruit (n = 6). *Indicates significant ($P \leq 0.05$) differences between the mean values of control and irradiated fruits at the same time interval. Same letter on the bar for control and irradiated fruits indicates no significant differences over time ($P \leq 0.05$). Horizontal line indicates the mean value on the day of harvest. nd means 'not detected'.

control fruits at 4 d after harvest. This may be due to reduced respiration immediately after irradiation (98). However, irradiation enhances the respiration at later stages of storage (98) and ethanol accumulation in irradiated fruits in the later stages in the present study may be due to the low O₂ concentration in the tissue, which results from rapid respiration (114). Arevalo-Garlaza et al. (115) also observed that respiration rate and ethanol production in fruits irradiated at 100 and 150 Gy increased up to 7 and 9 d after treatment, respectively. Melons exposed to post-harvest irradiation have also shown marked increases in respiration rate and ethanol content (116). Ethanol content at 4 and 6 d after harvest in control fruit and only 6 d after harvest in irradiated fruit was higher than the day of harvest. This may be due to waxing of the fruit after harvest. Waxing of fruits increases the internal CO₂ content in the fruits and reduces the oxygen level leading to greater ethanol accumulation (117). Similar to other volatile compounds, a significant ($P \leq 0.05$) decline in ethanol content was observed in freeze dried control fruit compared to fruits at 4 and 6 d after harvest (113). Unlike other volatiles, ethanol content in freeze dried controls was not significantly different from the day of harvest. This may be due to differences in volatility of these compounds.

4.4.4 Influence of irradiation and freeze drying on limonoid aglycones

Limonin and nomilin content was significantly reduced ($P \leq 0.05$) in irradiated freeze dried pulp compared to freeze dried controls (Fig. 23). However, no significant differences were observed in obacunone for irradiated

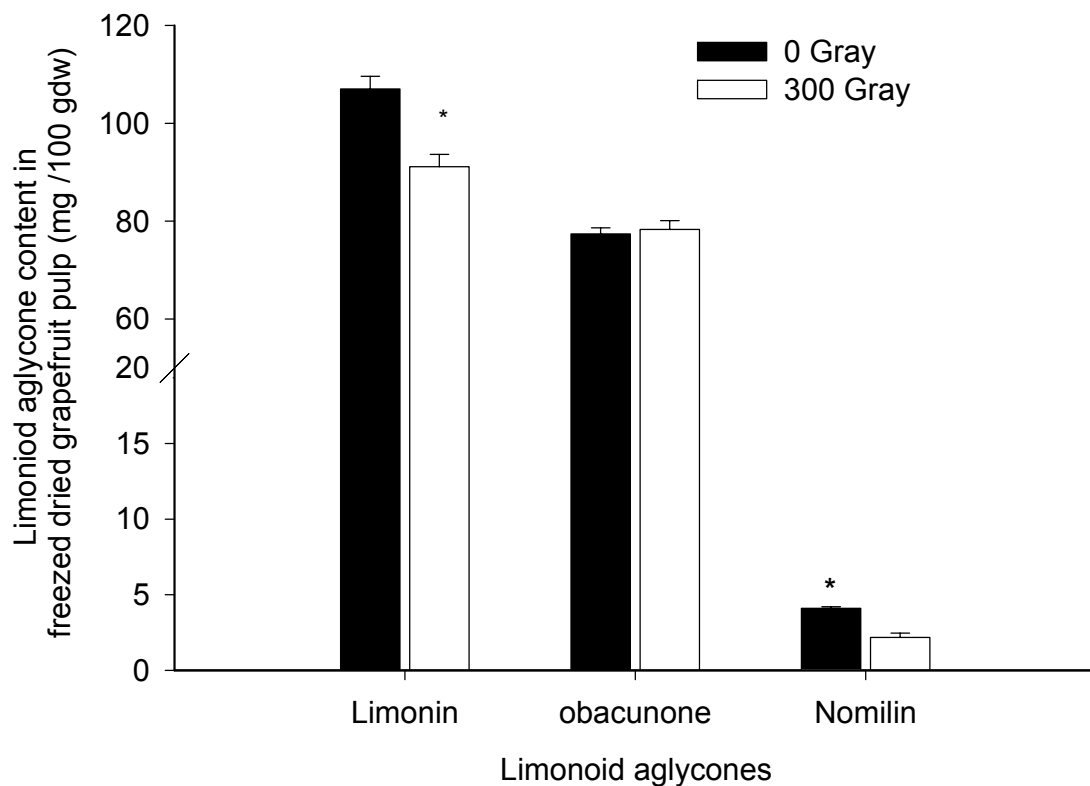


FIGURE 23 Limonin, obacunone, nomilin content (mg / 100 gdw) of freeze dried 'Rio Red' grapefruit pulp (n = 3). *Indicates significant ($P \leq 0.05$) differences between the compound mean values for control and irradiated freeze dried grapefruit pulp.

and control fruits. To the best of our knowledge this is the first study to demonstrate effect of irradiation and freeze drying on limonin, obacunone and nomilin.

In summary, our results suggest that postharvest irradiation and freeze drying have significant effects on the bioactive components of grapefruit. Thus, it is important to take the postharvest processes into consideration when fruit and vegetable based dietary supplements are being developed. The major difficulty in phytochemical research is obtaining valid results due to the high chemical and biochemical lability of many phytochemicals. In order to obtain accurate results for the chemopreventive ability of fruit and vegetable components, it is essential to use suitable methods for preservation of bioactive compounds.

CHAPTER V

**SUPPRESSION OF COLON CANCER DEVELOPMENT IN SPRAGUE
DAWLEY RATS BY NATURAL AND IRRADIATED GRAPEFRUITS AND
THEIR FUNCTIONAL COMPOUNDS**

5.1 Synopsis

Since the antiproliferative activity of citrus functional components against breast cancer has been reported, we hypothesized that citrus may also be protective against colon cancer. To test this, rats (n=100) were provided with one of five diets: control diet (AIN-76 containing 60 g/kg pectin), 13.7 g/kg grapefruit pulp powder (GFPP; providing 200 mg/kg naringin), 13.7 g/kg irradiated grapefruit pulp powder (IGFPP; 300 Gy, ^{137}Cs , a proposed treatment against fruit flies), naringin (200 mg/kg) or limonin (200 mg/kg). Following saline or azoxymethane injection (15 mg/kg) during the 3rd and 4th wk after starting the diets, colons were resected (6 wk post 2nd injection) and evaluated for aberrant crypt (AC) formation and cell proliferation. Total number of AC (59.9, 60.3, 62.1, 58.5% of control; $P = 0.02$), number of high multiplicity AC foci (ACF; 41.0, 48.2, 48.2, 34.5% of control; $P = 0.01$), and proliferative index (78.4, 85.5, 82.9, 81.7% of control; $p=0.02$) were lower in GFPP, IGFPP, naringin, and limonin treated rats, respectively. Only GFPP and limonin caused a smaller ($P = 0.03$) proliferative zone. All experimental diets significantly ($P = 0.02$) enhanced apoptotic index compared to the basal diet in AOM injected rats. Reduced proliferation and enhanced apoptosis might have contributed to a reduced

number of HMAF in rats on experimental diets. These results provide in vivo evidence for a potential role of grapefruit pulp and its functional components against the promotion stage of colon cancer.

5.2 Introduction

Colon cancer is the second leading cause of death from cancer in developed nations (2). However, a large body of evidence from epidemiological studies over the past four decades has shown that diets rich in fruit and vegetables are protective against a number of different cancers, including colon cancer (1). Mortality due to non-hereditary colon cancer seems to be preventable with appropriate changes in diet and modifiable non-dietary factors, such as smoking (118).

Areas with four or more aberrant crypts are termed as high multiplicity aberrant crypt foci (HMAF). HMAF correlate particularly well with the incidence of colorectal adenomas and carcinomas in AOM-induced rat models (119,120). Proliferation and apoptosis are the two most important processes in colonic epithelial cell maintenance. Abnormal cell proliferation is suggested to play an important role in multistage carcinogenesis, including colon tumorigenesis (121).

Recent experiments with animal models have provided direct evidence of the protective effects of fruits and vegetables on colon cancer development. Aberrant crypt foci (ACF), a surrogate biomarker for colon cancer induction by a colon specific carcinogen azoxymethane (AOM), was significantly reduced

through dietary administration of freeze dried vegetables (peas, spinach, sprouts and broccoli) in the rat model (48). Studies have also reported the chemoprotective effects of tomato and onion on ACF formation in AOM-induced colon cancer (122,123). Even certain isolated compounds from fruit and vegetables also confer protective effects against AOM-induced colon cancer (5).

'Rio Red' grapefruit are a rich source of bioactive constituents, which may serve as chemopreventive agents (4), in addition to having other beneficial effects on human health. These constituents include flavonoids, limonoids and their glucosides, vitamin C, folic acid, carotenoids (lycopene and beta-carotene), coumarin-related compounds (auraptene), high quality soluble fiber and potassium. In fact, a grapefruit phytochemical, limonin, has been found to significantly reduce the incidence of colonic adenocarcinoma induced by AOM in male F344 rats (5).

This study evaluated the chemopreventive ability of natural grapefruit, irradiated (300 Gy – a proposed quarantine treatment) grapefruit and two isolated compounds (naringin and limonin) against chemically induced colon carcinogenesis at the promotion stage of colon carcinogenesis. To evaluate whether these treatments had any protective effects, ACF, proliferative index and apoptotic index were investigated.

5.3 Materials and Methods

5.3.1 *Animal and study design*

The animal use protocol was approved by the University Laboratory Animal Committee of Texas A&M University and conformed to NIH guidelines. This experiment utilized a 5X2 factorial design comprised of five diets (Table 9) and two treatments: Azoxymethane (AOM; Midwest Research Institute, Kansas City, Missouri, USA) or saline. One hundred male Sprague-Dawley rats (Harlan Sprague-Dawley, Houston, TX) were individually housed and maintained in a temperature and humidity controlled animal facility. The rats were acclimated for 4 d prior to receiving the defined diets for 10 wk. Rats were stratified by body weight so that mean initial body weight was similar among the five diet groups and the two treatment groups.

The control or irradiated freeze dried grapefruit pulp powder was added to a modified AIN-76A diet with the aim of bringing the naringin concentration in the diet to 200 mg/kg. Grapefruit pulp (-80⁰C) devoid of seeds, albedo and flavado was freeze dried at NASA, Shuttle and ISS Food Systems, Johnson Space Center, Houston, TX to improve the stability and uniformity of the pulp.

Freeze dried pulp was stored under nitrogen at -20⁰C before the preparation of experimental diets. Freeze dried grapefruit pulp was powdered before the preparation of the experimental diets to enable proper mixing. Purified naringin or limonin (200 mg/kg) were added to the remaining two diets.

TABLE 9
Composition of the diets

Diet Components	Basal Diet	Basal Diet + GFPP*	Basal Diet + IGFP**	Basal Diet + Naringin***	Basal Diet + Limonin****
g/100g					
Dextrose ¹	51.06	49.87	49.87	51.04	51.04
Casein ¹	22.35	22.35	22.35	22.35	22.35
Pectin ²	6.00	5.82	5.82	6.00	6.00
Mineral Mix ¹	3.91	3.91	3.91	3.91	3.91
Vitamin Mix ³	1.12	1.12	1.12	1.12	1.12
D,L-methionine ¹	0.34	0.34	0.34	0.34	0.34
Choline Bitartrate ³	0.22	0.22	0.22	0.22	0.22
Corn Oil ⁴	15.00	15.00	15.00	15.00	15.00
Treatment components	0.00	1.37	1.37	0.02	0.02

*Grapefruit Pulp Powder; **Irradiated Grapefruit Pulp Powder; ***Sigma-Aldrich Corporation, St. Louis, MO; ****Limonin with 95% or greater purity (supplied by Dr. B. S. Patil).

¹Bio-Serv, Frenchtown, NJ.

²Danisco Cultor, New Century, KS.

³Harlan, Indianapolis, IN.

⁴Traco Labs, Champaign, IL.

Differences in pectin content due to grapefruit pulp powder supplementation were corrected by reducing the amount of added pectin in the grapefruit supplemented diets. Diets were stored at -20°C through the experimental period. After 4 d of acclimation, the rats were provided with the experimental diets for 10 wk. Rats received fresh food everyday. Fresh drinking water (*ad libitum*) was given on alternate d. Animals were injected with AOM (15 mg/kg body weight), a colon carcinogen, or saline, 3 wk after starting the experimental diets. One week after the first injection, a second AOM injection was given to the rats. For each animal, 48-hr diet intakes and body weights were recorded before the injections and at termination.

5.3.2 Tissue sample collection

Rats were euthanized 10 wk after starting the diets using CO₂ and the entire colon were removed and cleaned with RNase free PBS. One centimeter sections of the most proximal and distal portions of the colon were fixed in 4% PFA and 70% ethanol. The remaining midsection of the colon was cut open vertically; one half was used for an ACF assay and the other half were used for extraction of RNA and protein.

5.3.3 Aberrant crypt foci assay

Aberrant crypt foci (ACF) represent single, or a cluster of, morphologically altered crypts in AOM-injected rodents colonic mucosa (11,16,47,124). One-half of the colon mid-section to be used for determination of ACF, was placed in a folded piece of Whatman #1 filter paper and fixed in 70% ethanol for 24 h. Fixed

colons were dipped in a 0.5% solution of methylene blue in distilled water for 45 s and placed on a microscope slide with the mucosal surface up. Using a light microscope at 40X magnification, ACF and HMACF were distinguished from the surrounding “normal-appearing” crypts using standard criteria (5,11).

5.3.4 Proliferating cell nuclear antigen assay

Cell proliferation was measured using the proliferating cell nuclear antigen (PCNA) assay described by Zhang et al. (125) with a monoclonal antibody (anti-PC-10, Signet Lab). The bound primary antibodies were detected by applying a peroxidase-conjugated antibody to biotinylated anti-mouse immunoglobulin using a Vectastain ABC Elite Kit (Vector Lab). The intact antibody-antigen complex was made visible by placing slides into a solution containing 0.5 mg diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical) per mL PBS (Sigma Chemical) plus 0.05% H₂O₂ (added immediately before staining). Negative slides were prepared by substituting anti-PC 10 with same volume of PBS. Total number of stained proliferating cells, the height stained proliferating cell and total cells (crypt column height) for each crypt column was determined in 25 crypt columns. Proliferative index (number of stained proliferating cells/crypt column height) and proliferative zone (height stained proliferating cell/ crypt column height) were calculated.

5.3.5 Apoptosis assay

The TUNEL assay which detects 3'-OH termini of DNA fragments, were performed to determine the effect of diet on apoptosis (126). Colon tissue sections were pretreated with proteinase K (Ambion, Austin, TX) for 3 min at 37°C. A working solution of TdT-reaction buffer, with a ratio of 1:80, was applied to the sections and incubated for 1 h in a pre-warmed 37°C humidified chamber. After the incubation, methyl green was used to counter stain the tissue sections. Chemical positive control sections were prepared by nicking DNA with DNase I (deoxyribonuclease I) for 5 min. Rat colon sections obtained 12 h after injecting AOM served as biological positive control, as this time point invariably contains numerous apoptotic cells. PBS was substituted for 'TdT' in the working solution for developing negative control tissue sections. Total number of stained apoptotic cells, and total cells for each crypt column (crypt column height) was determined in 50 crypt columns. Apoptotic index (stained apoptotic cells/crypt column height) was calculated.

5.4 Results

5.4.1 General observations

Reverse phase HPLC analysis of experimental diets indicated that experimental constituents or compounds concentration in the diets were comparable with the intended concentration (200 mg/kg). Naringin concentration in GFPP, IGFP, and naringin diets was 196, 192, and 196 mg/kg, respectively. Limonin concentration in the limonin diet was 194 mg/kg. Food consumption and

body weight gain of rats in the various diet groups are shown in Table 10. Food intake and body weight gain did not differ among the groups suggesting that experimental diets were tolerated and supported normal growth in rats with out any adverse effects.

5.4.2 Aberrant crypt foci (ACF)

Data on total AC and HMAF are presented in Fig. 24 and 25. All rats injected with AOM developed AC. Rats receiving the basal diet had 185 ± 19.7 total AC and 13.9 ± 1.8 HMAF, respectively. Experimental diets reduced the total number of AC ($P = 0.02$) and HMAF ($P = 0.01$) compared to the basal diet. Saline injected rats showed no microscopically observable changes in colonic morphology.

5.4.3 Proliferation

The PCNA labeling indices for the rats from basal and experimental diets are presented in Fig. 26. Proliferative index was significantly ($P = 0.005$) higher in AOM injected rats receiving the basal diet, compared to the saline injected rats. Experimental diets reduced ($P = 0.02$) the proliferative index in AOM-injected animals compared to the AOM-injected rats consuming the basal diet. Proliferative zone was ($P = 0.008$) larger in AOM-injected rats consuming the basal diet, compared to the saline-injected rats (Fig. 27). Only diets containing natural grapefruit pulp powder and limonin prevented ($P = 0.03$) the AOM-induced expansion ($P = 0.008$) of the proliferative zone that occurred with the

TABLE 10

Mean food intake and weight gain of dietary intervention groups during 10 wk on experimental diets for both AOM and Saline injected rats

	Mean Food	Initial	Weight
Treatments	Intake (g)*	Weight (g)*	Gain (g)*
Saline			
Basal Diet	17.4 ± 0.43	57.3 ± 1.8	322.1 ± 7.5
GFPP	16.8 ± 0.43	54.7 ± 1.8	301.7 ± 7.5
IGFPP	17.2 ± 0.43	57.5 ± 1.8	301.7 ± 7.5
Limonin	17.9 ± 0.43	56.9 ± 1.8	313.9 ± 7.5
Naringin	17.0 ± 0.43	54.7 ± 1.8	324.3 ± 7.5
AOM			
Basal Diet	17.2 ± 0.43	53.6 ± 1.8	300.6 ± 7.5
GFPP	16.8 ± 0.43	57.0 ± 1.8	306.4 ± 7.5
IGFPP	17.4 ± 0.43	54.3 ± 1.8	304.9 ± 7.5
Limonin	17.3 ± 0.43	54.8 ± 1.8	312.9 ± 7.5
Naringin	17.2 ± 0.43	56.4 ± 1.8	322.6 ± 7.5

Data presented as LS means ± SEM.

*No diet or treatment effect was observed at $P \leq 0.05$.

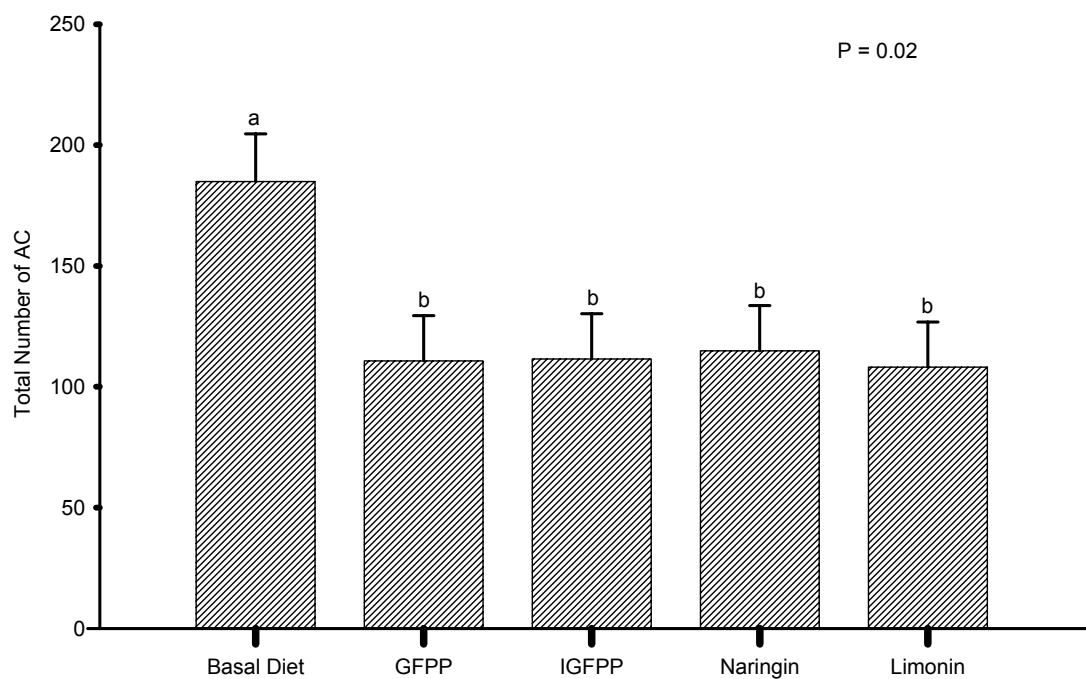


FIGURE 24 Aberrant crypt (AC) formation in AOM injected rats. Total AC were determined by microscopic examination (40 X) of the mucosal surface (half colon) stained with methylene blue. Different letters represent significant differences ($P = 0.02$) among the diets. Each bar indicates the least square mean of 10 rats \pm SEM (19.7). GFPP, Grapefruit Pulp Powder; IGFP, Irradiated Grapefruit Pulp Powder.

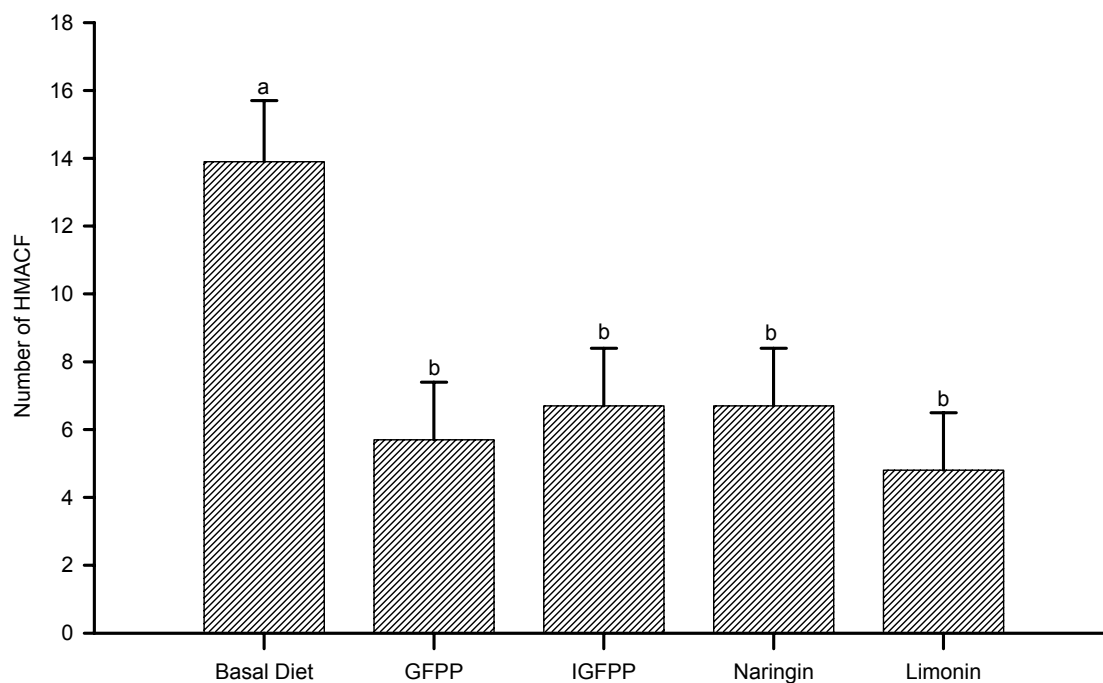


FIGURE 25 High multiplicity aberrant crypt foci (HMACF) formation in AOM injected rats. HMACF were determined by microscopic examination (40 X) of the mucosal surface (half colon) stained with methylene blue. Different letters represent significant differences ($P = 0.02$) among the diets. Each bar indicates the least square mean of 10 rats \pm SEM (1.8).

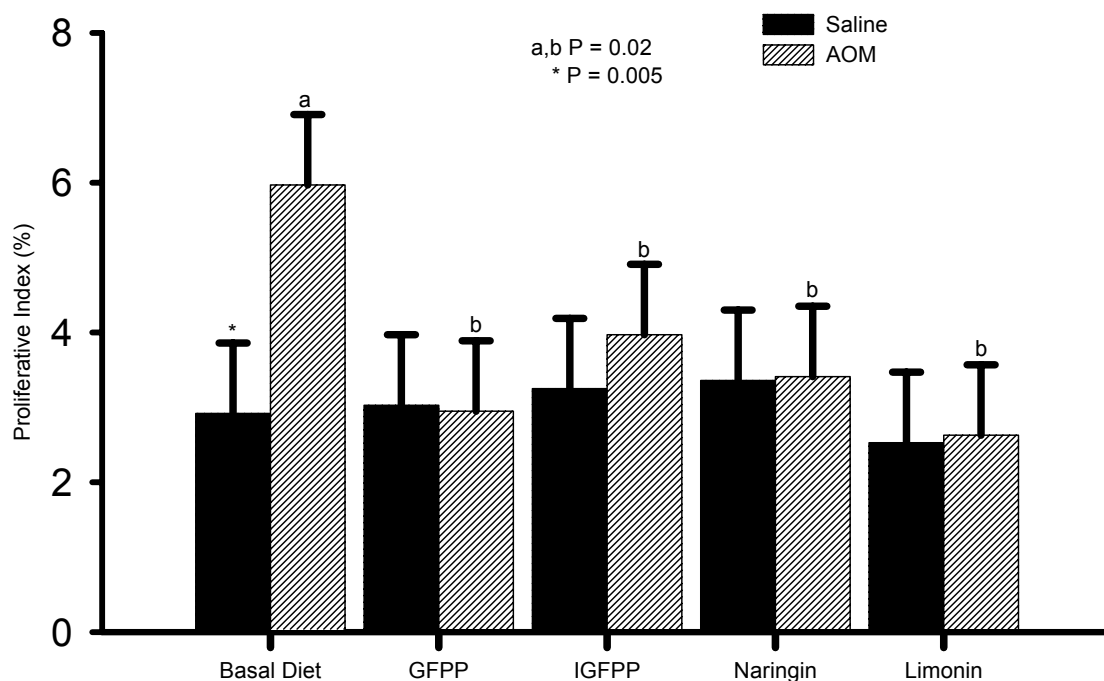


FIGURE 26 Effect of experimental diets on proliferative index in AOM- and saline-injected rats. Proliferation index was calculated as the total stained proliferating cells divided by the crypt column height. Different letters on the bars for AOM injected animals represents significant differences ($P = 0.02$) among the diets. *Indicates a significant ($P = 0.005$) difference in the mean values of saline- and AOM-injected animals consuming the same diet. Each bar indicates the least square mean of 10 rats \pm SEM (2.36).

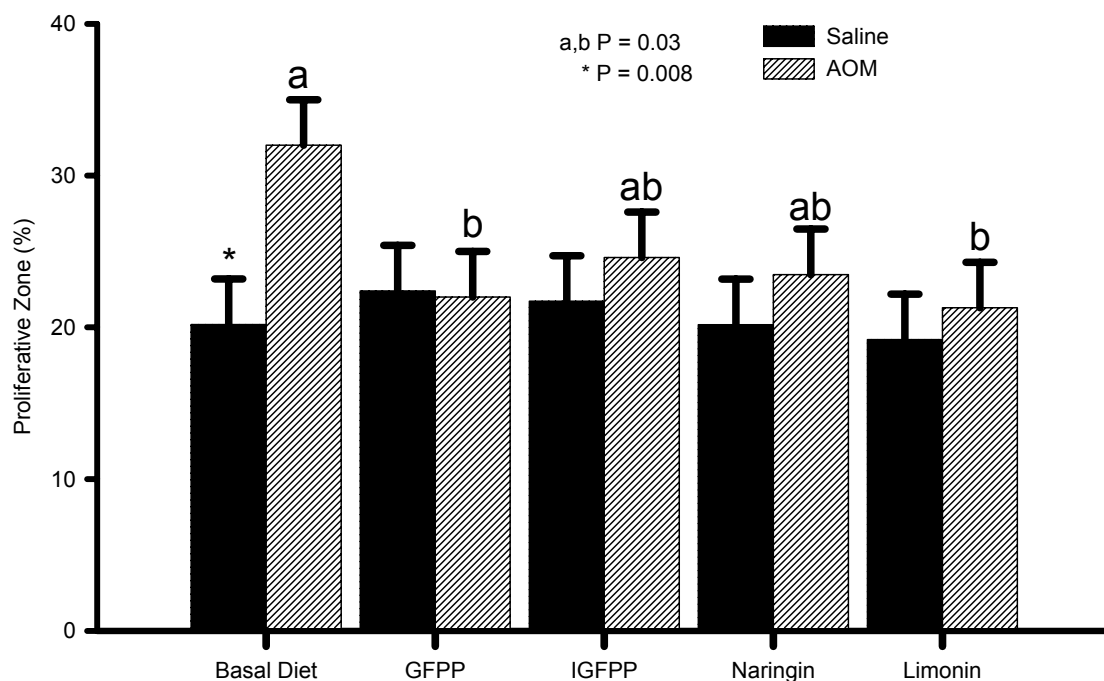


FIGURE 27 Effect of experimental diets on proliferative zone (% of crypt column height) in AOM- and saline-injected rats. Proliferative zone was calculated as the position of the highest stained proliferating cell divided by the crypt column height. Each crypt was visually divided into half and the number of stained cells and number of cells per crypt column were counted for 25 crypts. Different letters on the bars for AOM-injected animals represent significant differences ($P = 0.03$) among the diets. *Indicates a significant ($P = 0.008$) difference in the mean values of saline- and AOM-injected animals for the same diet. Each bar indicates the least square mean of 10 rats \pm SEM (3.0).

basal, IGFP and Naringin diets. Proliferative index or proliferative zone were not significantly ($P \leq 0.05$) different among diet groups in saline injected rats.

5.4.4 Apoptosis

Experimental diets do not significantly ($P \leq 0.05$) influence apoptotic index of saline injected rats (Fig. 28). However, experimental diets increased ($P = 0.02$) apoptotic index, compared to the basal diet, in AOM-injected rats. Apoptotic index in AOM injected rats on GFPP, naringin, and limonin diets were ($P = 0.01$) greater than that of saline-injected rats consuming those diets.

5.5 Discussion

This study was designed to determine whether grapefruit and isolated grapefruit compounds (naringin – a flavonoid, and limonin – a limonoid) would suppress the ‘promotion’ stage of AOM-induced colon carcinogenesis in Sprague Dawley rats. The study also addressed the question of whether radiation treatment of grapefruit influences the responses. Results from this study clearly indicate that dietary administration of GFPP, IGFP, naringin and limonin significantly inhibits AOM-induced AC formation in rats without causing any adverse effects on food intake or growth.

HMACF correlate particularly well with the incidence of colorectal adenomas and carcinomas in AOM-induced rat models (119,127). Interestingly, the number of HMACF per colon was also reduced ($P = 0.01$) by the experimental diets in this study. These findings suggest that grapefruit, naringin and limonin may suppress chemically induced colon carcinogenesis.

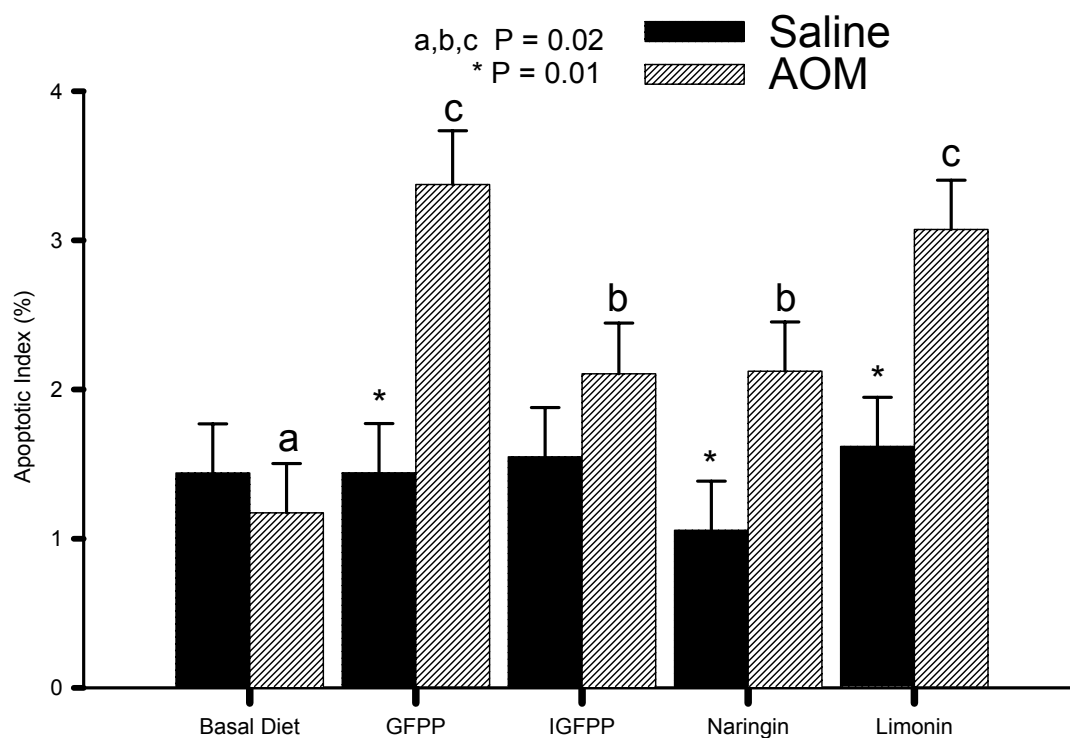


FIGURE 28 Effect of experimental diets on apoptotic index in AOM- and saline-injected rats. Apoptotic index was measured using TUNEL assay on paraffin embedded tissues. Fifty crypt columns were scored and the apoptotic index was calculated as the number of apoptotic cells divided by the number of cells per crypt column. Different letters on the bars for AOM-injected animals represent significant differences among the diets ($P = 0.02$). *Indicates a significant ($P = 0.01$) difference in the mean values of saline- and AOM-injected animals for the same diet. Each bar indicates the least square mean of 10 rats \pm SEM (0.36).

Recent study reports that natural grapefruit juice suppresses carcinogen induced colon DNA damage in rats (4). These results suggest that grapefruit also would suppress the 'initiation phase' of colon carcinogenesis. In another study, feeding rats with a grapefruit flavonoid extract (0.1-0.4%) resulted in an increased mass of intestinal contents in caecum, an increase in the intestinal wall mass and terminal pH in caecum, as well as an increase in the α -galactosidase activity of caecal microflora. However, feeding of grapefruit flavonoid extract had no influence on diet intake and body weight gain, but slightly increased the anti-oxidative potential of serum (128). Nguyen and Canada (129) reported that dietary citrus flavonoids may modulate colonic secretions, possibly through direct interaction with intracellular secretory pathways in human colonic T84 cells.

Limonin, abundant in orange and grapefruit, inhibited lung tumor formation in mice, and topical application of limonoids was found to inhibit both the initiation and promotion phases of carcinogenesis in the skin of mice (41). Furthermore, studies conducted in Japan showed that feeding orange juice, rich in flavonoids and limonoids, significantly inhibited AOM-induced colon cancer in male Fisher 344 rats (42). In a recent study, Fisher rats were injected with AOM (20 mg/kg body weight, once a week for 2 wk) and obacunone and limonin were fed in the diet at dose levels of 200 or 500 mg/kg for 4 wk during initiation or post-initiation. Interestingly, both the citrus chemicals significantly inhibited ACF

formation (55-65% reduction by 'initiation' feeding, 28-42% reduction by 'post-initiation' feeding). However, no significant differences were observed between rats fed with 200 or 500 mg/kg of limonin or obacunone (5).

Naringin, a flavanone glucoside, is rich in grapefruit, but naringenin, the aglycone of naringin is not abundant. However, Erlund et al. (33) reported relatively high concentrations of naringenin ($6.0 \pm 5.4 \mu\text{mol/L}$) in human plasma after the ingestion of grapefruit juice (8 mL/kg). These results suggest that naringin may be converted to naringenin in the intestine and then absorbed into the body. Like most flavonoids, naringenin has metal chelating, antioxidant and free radical scavenging properties (130), and has been reported to offer some protection against mutagenesis (131), lipid peroxidation (132), and excessive in vitro cell proliferation (133). Naringin also inhibits COX-2 activity, which plays a pivotal role in colon carcinogenesis (38). However, to our knowledge this is the first study to show the effect of naringin on AOM-induced colon cancer in animal models.

Several explanations for the suppression of HMA CF induced by AOM with dietary administration of GFPP, IGFPP, naringin and limonin are considered. Animal and human studies on cancer development suggest that proliferation and apoptosis are the two most important determinants or mediators in the process of ACF formation. Subjects with increased risk of colon cancer have a larger proliferative zone and a higher labeling index than subjects at low risk for colon cancer (24). Animals treated with a colon carcinogen also

have a larger proliferative zone and labeling index than animals treated with vehicle (23). The current study also showed that AOM-injected rats receiving the basal diet had a significantly higher proliferative index ($P = 0.005$) and larger proliferative zone ($P = 0.008$) compared to saline-injected rats. However, AOM-injected rats receiving the experimental diets had a significantly ($P = 0.02$) lower proliferative index compared to rats on basal diet. Only diets containing natural grapefruit pulp powder and limonin prevented ($P = 0.03$) the AOM induced expansion ($P = 0.008$) of the proliferative zone that occurred with the basal diet.

These results indicate that oxidative stress induced by grapefruit irradiation may cause a slight reduction in chemopreventive ability of grapefruit, and that limonin is more potent than naringin in suppressing expansion of the proliferative zone. Tian et al. (25) reported that the growth-inhibitory effects of limonoids and a limonoid glucoside mixture against breast and stomach cancer cells were significant, and the antiproliferative activity of the different citrus limonoids was also dose and time dependent. Recent studies show that naringenin suppresses the phosphoinositide 3-kinase (PI3K) activity in adipose cells (134). A large body of research data suggests that members of the PI3K family can also be considered as oncogenes because they control cell cycle progression and differentiation (135-137). GFPP containing naringin, limonin and other bioactive compounds might have created a greater antiproliferative effect in AOM-injected rats compared to the diets containing 200 mg/kg naringin.

In the present study all experimental diets induced apoptosis in AOM injected rat colons. Other studies also document that diets rich in hesperidin, quercetin and rutin (citrus flavonoids) induce apoptosis in colonic tumors and suppress AOM-induced colon carcinogenesis (28,87,138). Tian et al. (25) reported that citrus limonoids could induce apoptosis in the MCF cell line. The apoptotic pathway in colon cancer cells can be regulated by cyclooxygenase-2 (COX-2) expression (139). COX-2 specific inhibitors have been shown to inhibit chemically induced carcinogenesis in rodent species (140) and in humans . Citrus flavonoids (naringin, hesperidin, and quercetin) were shown to suppress the lipopolysaccharide (LPS)-induced COX-2 production in vitro (38,141-143). It is possible that suppression of COX-2 expression by grapefruit bioactive compounds would have resulted in enhanced apoptosis in rats fed with experimental diets.

In conclusion, quarantine doses of irradiation had a minimal effect on grapefruit chemopreventive ability. Thus, natural or irradiated grapefruit, or isolated naringin and limonin compounds may serve as chemopreventive agents against colon cancer. However, only natural grapefruit and limonin only suppressed AOM induced expansion of proliferative zone and also enhanced apoptosis more effectively than other experimental diets indicating that natural grapefruit and limonin may serve as better chemopreventive agents compared to IGFP and naringin.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Reverse phase HPLC analysis of flavonoid content in 14 pasteurized not from concentrate (NFC) and 12 made from concentrate (MFC) orange juices showed that significant differences ($P \leq 0.05$) among the brands were observed for all of the prominent flavanone glucosides. MFC Orange juices contain higher flavonoid content compared to the pasteurized NFC orange juices. Within a brand, orange juice types containing added vitamin C and E were not superior in flavonoid content compared to those without the added vitamins. Hesperidin was found to be the major flavonoid followed by narirutin and didymin in orange juice. Naringin, narirutin, and poncerin were the major flavonoids in all brands of grapefruit juices. Didymin was considerably higher in NFC orange juices compared to MFC orange juices suggesting that didymin may be sensitive to concentration techniques. Interestingly, no correlation was observed between price and the total flavonoid content MFC orange juices and NFC grapefruit juices. However, significant negative correlation ($r = -0.49$; $P = 0.001$) was obtained for NFC orange juices.

Rio Red grapefruits (*Citrus paradisi* Macf.) were exposed to gamma irradiation from a ^{137}Cs source at 0, 150 and 300 Gy and then stored at 10°C for 36 d, followed by an additional 20 d at 20°C to simulate marketing conditions. Flavanone, terpenoid and quality (acidity and total soluble solids) were evaluated at regular intervals during storage. Results showed that irradiation and

storage significantly ($P \leq 0.05$) affected the bioactive compounds in grapefruit, however, the effect of storage was prominent. Irradiation differentially effected the flavanone content of pulp and peel. For example, fruits exposed to 300 Gy had significantly ($P = 0.01$) higher narirutin content in peel compared to the fruits exposed to 0 Gy irradiation. However, narirutin content in pulp was not effected by irradiation. Even though storage enhanced the d-limonene and myrcene content in all treatments, control fruit had higher terpenoid content at the end of the storage. In general, irradiation or storage had no considerable effect on total soluble solids, however, acidity reduced ($P \leq 0.05$) with the storage and 300 Gy irradiated fruits better retained the acidity at the end of the storage.

Bioactive compounds from fruits and vegetables are widely considered to be valuable for human health. However, the bioactive compounds of fruits and vegetables are influenced by postharvest treatments. This aspect has been mostly ignored or barely considered even in the contemporary nutritional and epidemiological studies due to limited scientific data on postharvest effects on bioactive compounds. The present study evaluated the effects of a proposed quarantine dose of irradiation (300 Gy), freeze drying and postharvest storage on bioactive grapefruit compounds such as flavonoids, carotenoids, ascorbic acid, limonoid aglycones, volatile compounds, and ascorbic acid. Bioactive compounds were analyzed with reverse phase liquid chromatography with the exception of volatile compounds which were analyzed using gas chromatography. Freeze dried pulp from irradiated grapefruit resulted in

enhanced flavonoid content (naringin and narirutin). Freeze drying reduced ($P \leq 0.05$) grapefruit lycopene content, however, the reduction ($P \leq 0.05$) in β -carotene content was significant ($P \leq 0.05$) in control fruit only. Ascorbic acid was not affected by irradiation and the levels after storage were comparable to the day of harvest in control fruit. Freeze drying and irradiation (6 d) reduced volatile compounds (d-limonene and myrcene) with the exception of ethanol. These results warrants the necessity of testing the effect of postharvest treatments like irradiation and processing effects on bioactive compounds in functional system as they have varied effects on different bioactive compounds of grapefruit.

Chemopreventive ability of natural grapefruit, irradiated grapefruit and isolated compound (naringin and limonin) were evaluated in AOM induced colon cancer model. Rats ($n=100$) were provided with one of five diets: control diet (AIN-76 containing 60 g/kg pectin), 13.7 g/kg grapefruit pulp powder (GFPP; providing 200 mg/kg naringin), 13.7 g/kg irradiated grapefruit pulp powder (IGFPP; 300 Gy, ^{137}Cs , a proposed treatment against fruit flies), naringin (200 mg/kg) or limonin (200 mg/kg). Following saline or azoxymethane injection (15 mg/kg) during the 3rd and 4th wk after starting the diets, colons were resected (6 wk post 2nd injection) and evaluated for aberrant crypt (AC) formation and cell proliferation. Total number of AC ($P = 0.02$), number of high multiplicity AC foci (ACF; $P = 0.01$), and proliferative index ($P = 0.02$) were lower in GFPP, IGFPP, naringin, and limonin treated rats, respectively. Only GFPP and limonin caused

a smaller ($P = 0.03$) proliferative zone. All experimental diets significantly ($P = 0.02$) enhanced the apoptosis compared to the basal diet in AOM induced rats. Reduced proliferation and enhanced apoptosis might have contributed to reduced number of HMAF in rats on experimental diets. Thus, quarantine doses of irradiation had minimal effect on grapefruit chemopreventive ability. Natural or irradiated grapefruit or isolated naringin and limonin compounds may serve as chemopreventive agents against the promotion stage of colon cancer. However, only natural grapefruit and limonin only suppressed AOM induced expansion of proliferative zone and also enhanced apoptosis more effectively than other experimental diets indicating that natural grapefruit and limonin may serve as better chemopreventive agents compared to IGFPP and naringin.

Thus, postharvest quarantine doses of irradiation slightly alter composition of bioactive compounds and in turn the marginally reduce the chemopreventive ability of grapefruit against promotion stage of colon cancer. These results warrant the necessity of testing the impact of post harvest treatments on fruits and vegetables chemopreventive ability as there is accumulating evidence suggesting that fruits and vegetables are beneficial for prevention of chronic disease such as cancer, heart diseases and diabetes.

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Publications

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